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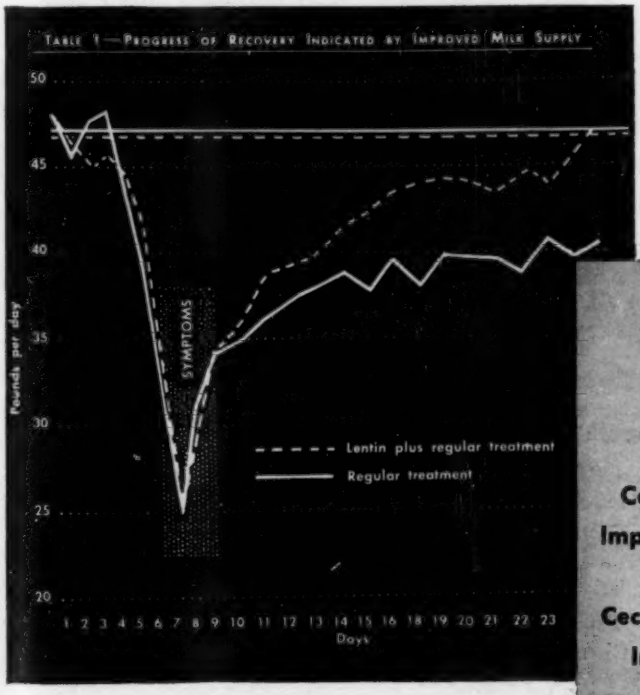
*Holm, G. C., A preliminary report of the use of Lentin in atonic indigestion of dairy cattle, Vet. Med. 39: 414-416, Nov. 1944.



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VOL. VII APRIL, 1946 NO. 23
In Two Parts — Part I

Changes in the Equine Larynx Associated with Laryngeal Hemiplegia

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Columbus, Ohio

SEVERAL FACTORS were considered in this study: changes in the muscles; changes in the nerves supplying the muscles of the larynx; and changes in the cartilages of the larynx (their shape, calcification, and relationship to each other).

It was necessary to study macroscopic and microscopic anatomy of the laryngeal cartilages, muscles, and nerves of many normal horses as well as those which showed the disease in order to be able to recognize variations from the usual normal condition. Careful dissection and histological examinations were performed, as well as the study of laryngeal movements by rhinolaryngoscopic examination and faradic stimulation.

In order to study the mechanism of "roaring" and to demonstrate that nerve lesions alone could be responsible for laryngeal hemiplegia the disease was produced artificially by recurrent neurectomy and local neural narcosis.

Roaring is produced by the power of the instreaming air against a larynx unable, from atrophy of intrinsic muscles, to open to air passage during inspiration.

In all, 174 equine larynges were examined. A complete autopsy was performed on 102 of the horses, and 160 tissue sections made for histopathological study.

From the Department of Veterinary Pathology, College of Veterinary Medicine, the Ohio State University.

For the entire work and references see "Changes in the Equine Larynx Associated with Laryngeal Hemiplegia," Master's Thesis, Library, The Ohio State University (1944).

EXPERIMENTAL PROCEDURE

The changes in the equine larynx associated with laryngeal hemiplegia were studied *ante mortem* in horses from the following sources: (1) horses showing symptoms of the disease purchased for study, (2) horses purchased by the veterinary clinic for use in operative surgery laboratory, and (3) horses brought to the veterinary clinic from the field artillery stables for euthanasia.

A complete autopsy was performed on 102 horses of the above-mentioned groups, and horses presented to the Department of Veterinary Pathology for autopsy.

Larynges were obtained from horses at autopsy, horses from fertilizer companies in Columbus and Defiance, Ohio, and horses used for dissection in the Department of Veterinary Anatomy, making a total of 174 larynges for examination.

Following the dissection of the nerve supply of the laryngeal muscles, sections were taken from the following points for microscopic study: (1) at the origin of the vagus, (2) along the course of the vagus, (3) the vagus near the point where the recurrent laryngeal nerves branch off, (4) both recurrents at their origin, (5) left recurrent nerve at the aortic arch, at the thoracic inlet, along its course, on each side of the thyroid gland, and at its termination; (6) the anterior laryngeal nerve external to and inside of the thyroid foramen, (7) the nervus anastomoticus, and (8) small branches of the recurrent nerves.

Transverse and longitudinal sections were made of all the intrinsic muscles of both sides of the larynx in order to compare the right and left muscles. The degree of atrophy of the

intrinsic muscles of the larynx was estimated by gross and microscopic examination.

Variations in length and diameter of the left and right recurrent and anterior laryngeal nerves were noted along their course from origin to termination.

The antemortem position of the laryngeal

Fig. 1—Larynx from a case which showed marked symptoms of laryngeal hemiplegia. (a) 90 per cent atrophy of left crico-arytenoideus dorsalis muscle; (b) normal right crico-arytenoideus dorsalis muscle; (c) right recurrent laryngeal nerve; (d) left recurrent laryngeal nerve; (e) cut end of esophagus; (f) left posterior projection of lamina of cricoid cartilage; (g) right posterior projection of lamina of cricoid cartilage.

cartilages was observed by use of the rhinolaryngoscope, and by direct observance through a longitudinal incision in the cricothyroid membrane.

After working out the possible pathways of impulses to or from the laryngeal muscles by naked eye and binocular dissections, faradic stimulation was utilized to discover (1) which nerves were actually conducting the impulses, (2) the exact function of each intrinsic muscle, and (3) the effect of variations in strength of stimuli. Secondary (induced) current supplied by Professional Battery No. 1. (Max Wocher & Son, Cincinnati, Ohio) was used for direct electrical stimulation of the nerves and muscles.

The laryngeal nerves were stimulated *intra vitam*, and immediately *post mortem*.

Variations in the degree of calcification of the laminae of the thyroid cartilage, as well as great variations in the length of the two posteriorly projecting portions of the lamina of the cricoid cartilage were noted. Since the crico-arytenoideus dorsalis muscle has its origin on the dorsal surface of the lamina of the cricoid cartilage, it was thought that a variation in the size of this lamina may have some relationship to the atrophy seen in that muscle.

Some horses showed symptoms of laryngeal hemiplegia while standing at rest while others had to be put to severe exertion before symptoms were shown. Horses were hitched to a capstan having a lever 13.5 ft. long, equipped with a brake. Then the horses were compelled to trot in the 85-ft. circle to determine if they would show "roaring" symptoms.

The sounds produced by the horses were recorded on audiodiscs by a recordiograph manufactured by the Bell Sound System, Inc., Columbus, Ohio. The microphone was held four inches anterior to the nostrils and high enough

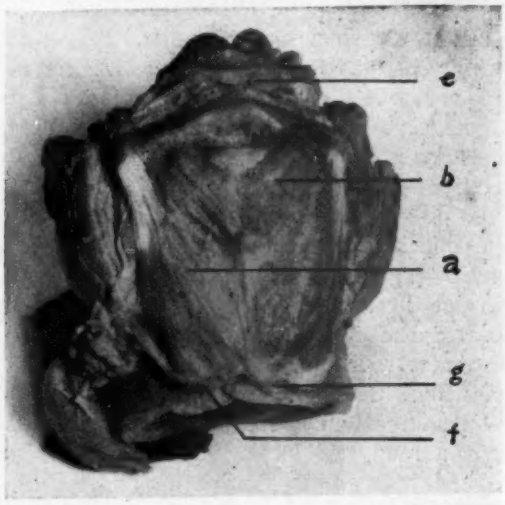

Fig. 2—Larynx from a horse which showed only mild symptoms of laryngeal hemiplegia, although marked muscle atrophy is apparent. (a) 90 per cent atrophy of left crico-arytenoideus dorsalis muscle; (b) normal right crico-arytenoideus dorsalis muscle; (e) cut end of esophagus; (f) left posterior projection of lamina of cricoid cartilage; (g) right posterior projection of lamina of cricoid cartilage.

to avoid expired air from the external nares from striking the microphone.

Calcification was observed in some of the laryngeal cartilages but was found to be unimportant. Calcification extending to the dorsal borders of the laminae of the thyroid cartilage was shown in 46.5 per cent of the larynges with atrophic muscles. The cricoid cartilages of eight larynges showed some calcification in the lamina, but no difference was noted between the right and

left sides. As most of the horses used in this study were over 6 years old, the calcification of the cartilages was expected to be more advanced than in younger horses.

A variation in the length of the posterior

Fig. 3—A normal larynx showing the right posterior projection of the cricoid lamina longer than the left. No "roaring" shown by the horse. (a) Right posterior projection of cricoid.

projections of the lamina of the cricoid cartilage was noticed after examining several larynges.

The relationship between the length of these projections and atrophy of the laryngeal muscles is shown in table 2; 42.3 per cent of all larynges had projections of equal length while the percentage of larynges with normal muscles (40.7%) and those with atrophic muscles (46.5%) in this group were about the same. More larynges had projections of equal length than either long right side or left side projections.

That this variation in the cricoid cartilage seemed to have had very little relationship to atrophy of the laryngeal muscles is shown by the data in table 2.

Table 3 gives the estimated percentage

TABLE 1—Relationship of Calcification of Thyroid Cartilages to Atrophy of Laryngeal Muscles

	Body only	1/4	1/2	3/4	4/4	Total
No. of larynges in each group.	10	48	32	16	40	146
Larynges in each group, %	6.8	32.8	21.2	10.9	28.0	..
No. of larynges with atrophic muscles	2	1	6	14	20	43
Larynges with atrophic muscles, % ...	4.65	2.32	13.95	32.55	46.5	..
No. of larynges with normal muscles	8	47	26	2	20	103
Larynges with normal muscles, % ...	7.7	45.6	25.2	1.94	19.4	..

Body only—refers to calcification only in the body of the thyroid cartilage.
1/4 = calcification extending from the body one-fourth of the distance to the dorsal border of the thyroid laminae.
1/2 = calcification extending half way to the dorsal border of the thyroid laminae.
3/4 = calcification extending three-fourths of the way to the dorsal border.
4/4 = refers to calcification extending from the body to the dorsal border of the laminae of the thyroid cartilage.

of atrophy of the left intrinsic laryngeal muscles as determined by gross and microscopic examination. Twenty-seven of these animals were exercised to determine whether they would show symptoms of laryngeal hemiplegia (*see* table 3).

From the amount of exercise given, no

TABLE 2—Relationship of Length of Posterior Projections of Cricoid Lamina to Atrophy of Laryngeal Muscles

	Equal length	Right longer than left	Left longer	Total
Number of larynges..	62	39	45	146
Larynges, %	42.3	26.0	30.8	...
Number with normal muscles	42	26.0	35	103
With normal muscles, %	40.7	25.2	33.9	...
Number with atrophied muscles	20	13	10	43
With atrophied muscles, %	46.5	30.2	23.2	...

TABLE 3—Per Cent of Atrophy of the Left Intrinsic Laryngeal Muscles

Larynx number	Roaring symptoms	Crico-arytenoi-deus dorsalis	Crico-arytenoi-deus lateralis	Arytenoi-deus trans-versus	Ventricu-laris	Vocalis	Crico-thyroideus
1	+ +	95	95	95*	75	50	0
2	+ + +	90	90	90	50	10	0
3	—	10	10	40	20	20	0
4	+	50	50	50	10	0	0
5	+ +	95	90	95*	60	50	0
6	—	10	10	20	0	0	0
7	—	20	10	10	0	0	0
8	—	50	20	50	0	0	0
9	—	80	50	90	10	0	0
10	+	90	90	90	50	10	0
11	—	10	0	10	0	0	0
12	—	40	20	40	0	0	0
13	—	50	20	40	0	0	0
14	—	10	0	20	0	0	0
15	—	10	0	50	0	0	0
16	—	10	10	20	0	0	0
17	—	20	10	20	0	0	0
18	—	20	10	30	0	0	0
19	—	40	20	50	20	10	0
20	—	50	40	50	10	10	0
21	?	80	50	90	10	20	0
22	+ +	95	90*	95	50	50	0
23	+ +	90	90	95	50	50	0
24	+	90	75	90	50	50	0
25	—	80	75	90	75	50	0
26	+	90	80	90	50	50	0
27	?	90	75	90	50	50	0
28	—	50	20	75	10	10	0
29	—	50	20	75	10	10	0
30	?	40	20	75	0	0	0
31	?	60	50	75	0	0	0
32	?	50	50	50	20	0	0
33	?	50	10	50	0	0	0
34	?	10	0	20	10	0	0
35	?	20	0	10	0	0	0
36	?	10	0	10	0	0	0
37	?	10	0	0	0	0	0
38	?	10	10	10	0	0	0
39	?	30	20	50	0	0	0
40	?	30	30	50	0	0	0
41	?	20	10	10	10	0	0
42	?	20	10	40	0	0	0
43	?	5	0	0	0	0	0
44	?	10	0	0	0	0	0
45	?	10	0	10	0	0	0
46	?	20	?	?	?	?	0
47	?	20	?	?	?	?	0

* = Ten per cent atrophy in the right half of the arytenoideus transversus also.
? = Not examined.
+ = Slight symptoms of laryngeal hemiplegia.
+ + = Intermediate symptoms shown.
+ + + = Marked symptoms shown.
— = No symptoms of laryngeal hemiplegia.

symptoms of laryngeal hemiplegia were shown in horses having less than 50 per cent atrophy of the left crico-arytenoideus dorsalis muscle.

Of the 174 larynges examined, 43 (27.04%) showed atrophy of one or more left laryngeal muscles.

Twenty-seven of the horses (out of 43 larynges showing atrophy) were worked before being destroyed to determine if symptoms of laryngeal hemiplegia would be shown. Nine of these 27 "roared," or in other words, only 25.8 per cent of the horses having atrophic laryngeal muscles actually showed symptoms of laryngeal hemiplegia. This substantiates earlier work and shows that there is a wide variation in muscular atrophy in horses that roar.

Larynges 5 and 6 showed 10 per cent atrophy of the right half of the arytenoideus transversus. Atrophy was neither observed in the right or left crico-thyroideus dorsalis, nor any of the intrinsic laryngeal muscles of the right side. No atrophy was present in any of the extrinsic muscles of the larynx.

Generally, atrophy was first noticed and was more severe in the left crico-arytenoideus dorsalis, and the left half of the arytenoideus transversus, followed in order by the left crico-arytenoideus lateralis, left ventricularis and left vocalis muscles. However, this order of degree of atrophy did not always hold true.

Larynx 9 in table 3 showed 90 per cent

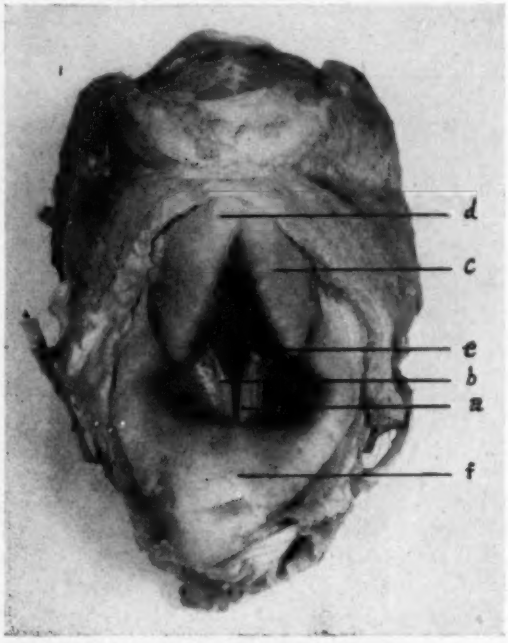

Fig. 4—Asymmetrical rima glottidis due to the dropped position of the left arytenoid cartilage. (a) Left vocal cord; (b) right vocal cord; (c) apex of left arytenoid cartilage; (d) apex of right arytenoid cartilage; (e) vocal process; (f) epiglottis.

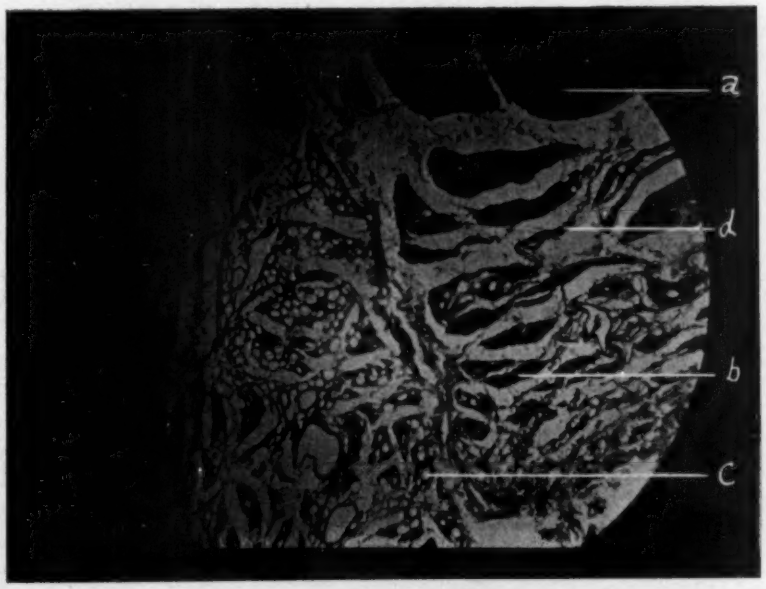

Fig. 5 — Atrophy of crico-arytenoideus lateralis. Hematoxylin and eosin. Transverse. Photomicrograph. x 80. (a) Normal muscle bundle; (b) atrophic muscle bundle; (c) vacuole; (d) perimysium.

atrophy in the left half of the arytenoideus transversus, and 80 per cent in the left crico-arytenoideus dorsalis; but no symptoms were shown by the living animal. There was some indication that the tone of the constrictor muscles, if normal, will partially adduct the arytenoid cartilage when the dilator muscle is atrophic and thus make inspiration more difficult.

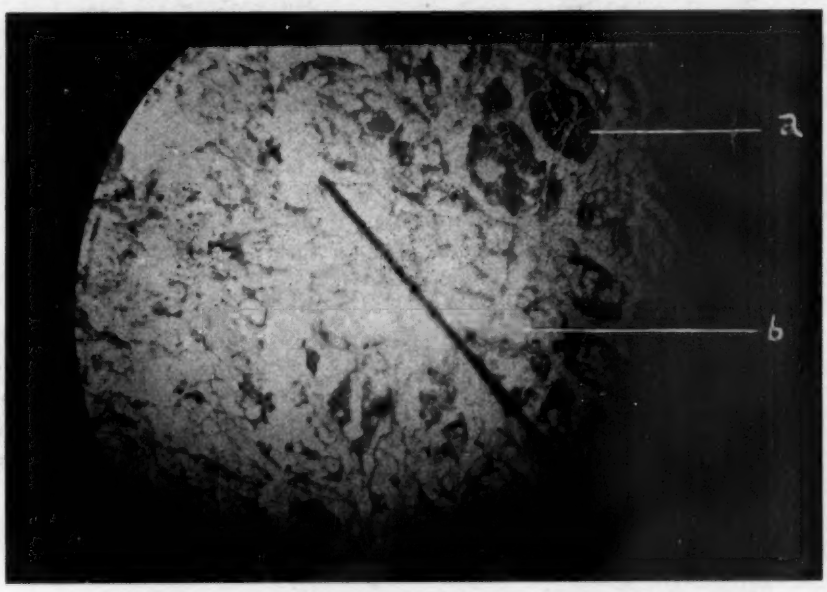

Fig. 6—Atrophy of crico-arytenoideus dorsalis muscle. Transverse. Photomicrograph. x 80. (a) Bundle of atrophic muscle fibers; (b) degeneration, vacuoles.

SUMMARY AND CONCLUSIONS

A total of 174 equine larynges was examined. A complete autopsy was performed on 102 of the horses, and 160 tissue sections were made for histopathologic examination.

The average distance between the vocal process of the right and left arytenoid cartilages of the equine larynx is 1.5 to 2.2 cm. (cadaver position). In cases of laryngeal hemiplegia, the left arytenoid cartilage hangs ventrally and nearer the median line of the larynx reducing the width of the rima glottidis at the vocal processes as much as 8 to 10 mm.

A variation in the length of the right and left posterior projection of the lamina of the cricoid cartilage was observed. Even though the crico-arytenoideus dorsalis has its origin on the cricoid lamina, there was no correlation between this variation in the cartilage and atrophy of the laryngeal muscles.

The average length of the left recurrent laryngeal nerve from its origin at the vagus in the thorax to its termination at the larynx was 97.6 cm. as compared to 76.0 cm. for the right recurrent. The greatest difference in length between the left and right recurrent nerves was 31.0 cm. No correlation existed between the length of the laryngeal nerves and the occurrence of laryngeal hemiplegia; 46.5 per cent of the larynges with atrophic muscles showed calcification of the thyroid cartilage extending to the dorsal borders of the laminae.

The average diameter of the fresh right recurrent nerve was 2.0 mm. as compared to 1.75 mm. for the left recurrent. In many cases of atrophy of the left laryngeal muscles, a marked diminution in size was noted along the course of the left recurrent nerve from its origin at the vagus to its termination. Often the left nerve was only

one-half the size of the right near the larynx.

Except for 10 per cent atrophy of the right half of the arytenoideus transversus and degeneration of a few nerve fibers in the right recurrent laryngeal nerve in two cases, all the muscular changes were confined to the left side. In only 4.6 per cent of the larynges showing muscular atrophy were there any demonstrable lesions on the right side. No larynx showed right unilateral muscular atrophy.

Horses having less than 50 per cent atrophy of the left crico-arytenoideus dorsalis muscle did not show symptoms of laryngeal hemiplegia from the exercise tests made.

In general, the left normal appearing (microscopic and macroscopic) crico-arytenoideus dorsalis muscle was less developed

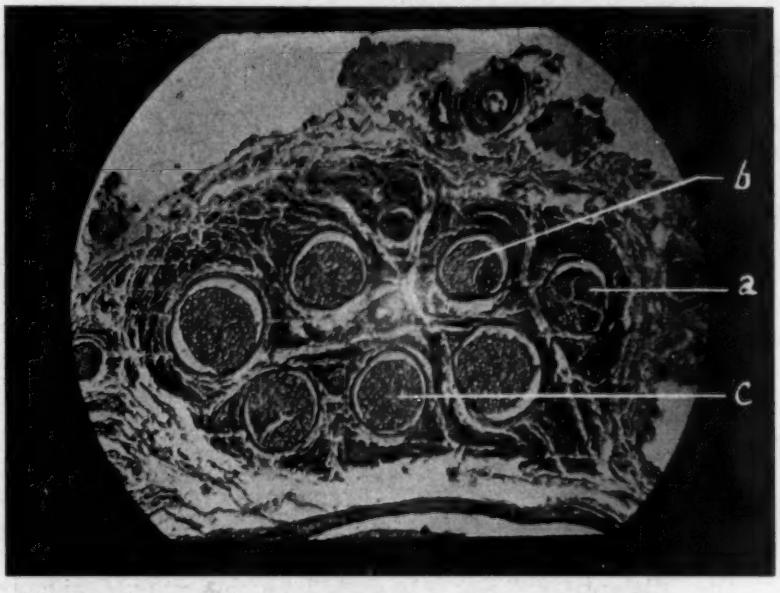

Fig. 7—Normal left recurrent laryngeal nerve. Transverse section taken at aortic arch. Hematoxylin and eosin. Photomicrograph. x 80. (a) Bundle of small finely myelinated fibers; (b) bundle of large heavily myelinated fibers; (c) bundle shown x 400 in fig. 8.

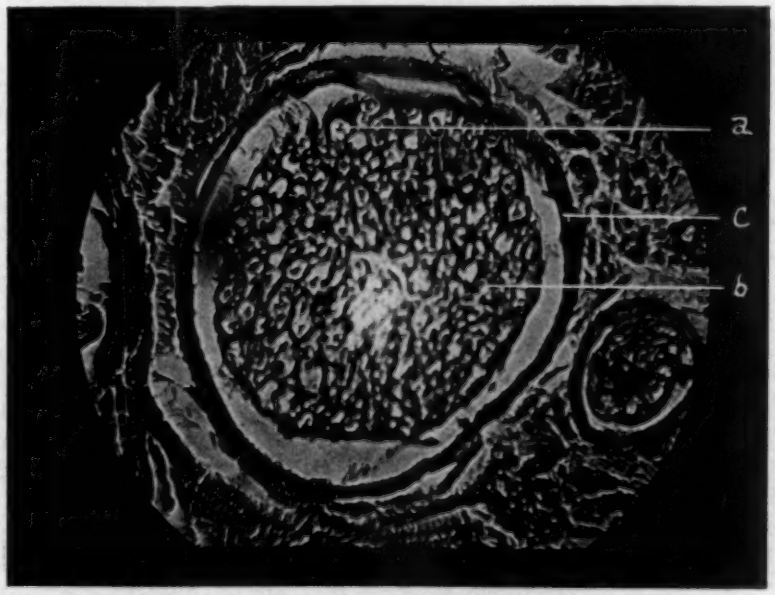

Fig. 8—Normal left recurrent nerve. Higher magnification of bundle (C) in fig. 7. Transverse. Photomicrograph. x 400. (a) Large heavily myelinated nerve fiber; (b) axis cylinder; (c) perineurium.

than the right, and few mature horses showed perfectly symmetrical positions of the arytenoid cartilages.

The chief microscopic changes in the muscles consisted of a reduction in the diameter of individual muscle fibers without changing the architecture, elimination of part of the sarcoplasm, loss of cross striations, and the appearance of more prominent central nucleoli in the nuclei. As a result of the myolysis and fatty degeneration some of the sarcolemma appeared as vacuoles. Some muscle fascicles were degenerated completely and replaced with connective tissue.

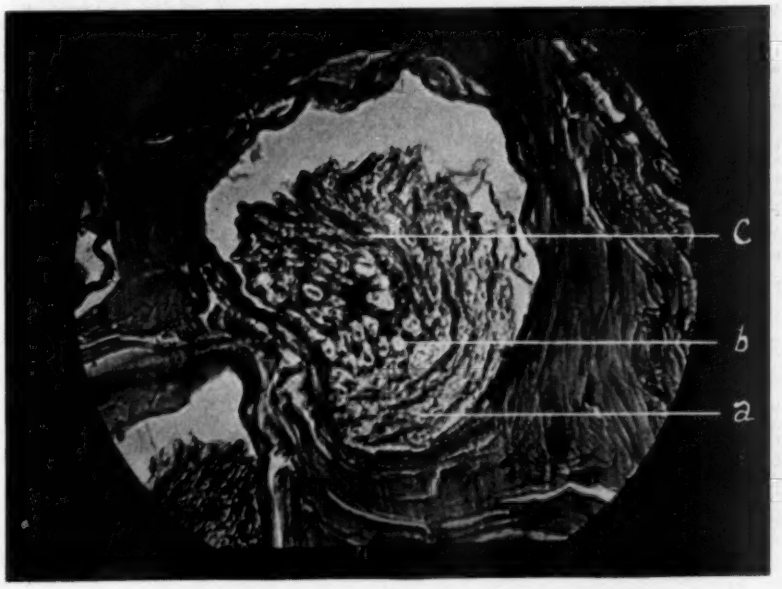

Fig. 9 — Degenerated left recurrent nerve bundle. Section 4 inches posterior to larynx. Photomicrograph. x 400. (a) Degenerated fiber; (b) disappearance of axis cylinder; (c) fibrosis.

Disintegration of the axis cylinders, with disappearance of the myelin sheaths, was observed in the left recurrent nerve which supplies the atrophic muscles. Proliferation of the cells in Schwann's sheath, endo-

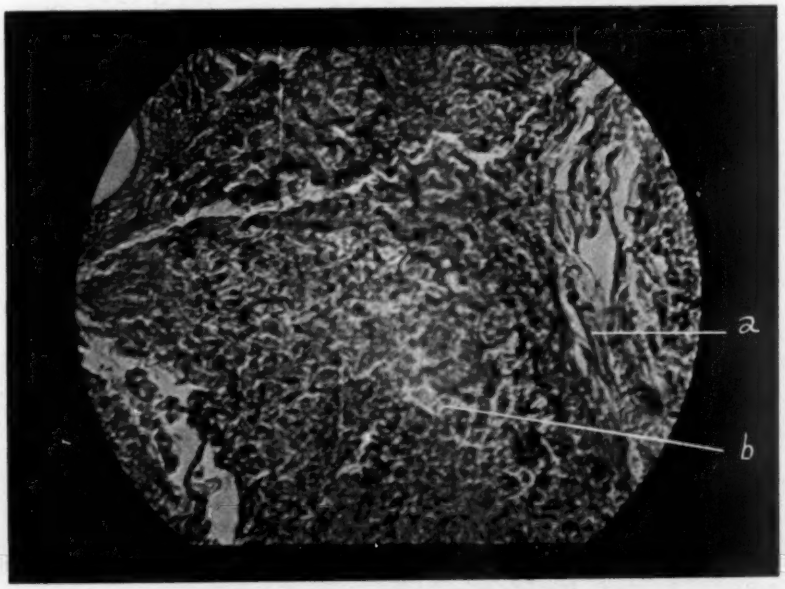

Fig. 10 — Degenerated left recurrent nerve bundle. Section at thoracic inlet. x 400. (a) Perineurium; (b) degenerated nerve fibers.

neurium, and perineurium finally resulted in complete fibrosis of the nerve.

Marked degeneration and fibrosis occurred in the peripheral portion of the left recurrent nerve. Sections of the nerve, progressing in a central direction, showed less degeneration until only a few fibers were involved at the thoracic inlet. Neuropathology was seldom seen in the left recurrent nerve in the region of the aortic arch.

The recurrent laryngeal nerve consists of six fascicles of large, heavily myelinated fibers and five fascicles of small, finely myelinated fibers. Degeneration was more often seen in the small fibers.

The left crico-arytenoideus lateralis and left half of the arytenoideus transversus showed atrophy nearly as often and as severe as the crico-arytenoideus dorsalis.

Only in cases of severe atrophy of the other muscles did the left ventricularis and vocalis muscles show atrophy.

When atrophy of the laryngeal muscles was apparent, there were degenerative changes noted in the recurrent nerve of the same side. Only two cases showed degenerative changes in the left anterior laryngeal nerve.

The proximity of the arytenoid cartilages and vocal cords was found to have a marked influence on the dyspnea. Atrophy of both the constrictor and dilator muscles of the larynx simultaneously resulted in milder symptoms of laryngeal hemiplegia than if the dilator alone was atrophic. It appeared that the tone of the constrictor muscles increased the power of adduction and thereby decreased the laryngeal capacity.

The recurrent nerves supply the motor impulses unilaterally to all the intrinsic laryngeal muscles except the crico-thyroideus. Stimulation of the recurrent nerve with a weak faradic current resulted in abduction (dilatation) of the arytenoid cartilage of the same side, while a strong current resulted in adduction (constriction) of the arytenoid cartilages.

The size of the thyroid glands and their relationship to the recurrent nerves could not be shown to have a bearing on laryngeal hemiplegia.

Even though neuropathic atrophy of the left laryngeal muscles was marked, none of the horses showed symptoms of a central nervous disturbance.

Of the 174 larynges examined, 43 (27.04%) showed atrophy of one or more left laryngeal muscles.

Only 25.8 per cent of the horses having varying degrees of atrophy of the laryngeal muscles actually showed symptoms of laryngeal hemiplegia from the amount of exercise given.

ACKNOWLEDGMENT

Sincere thanks are extended to Dr. Leonard W. Goss, chairman of the Department of Veterinary Pathology, for his wise counsel and generosity in materials and laboratory equipment.

A Contagious Disease of Cattle Associated with Leptospira

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DURING THE LATE summer of 1942, an epizoötic appeared in the cattle on three ranches in Brewster, Presidio, and Jeff Davis counties of Texas, which was not diagnosed at the time nor were efforts to show it to be of an infectious nature successful. A study of a more recent outbreak and of several sporadic occurrences have proved that this is an infectious and contagious disease, that the microscopic pathology resembles that recognized for leptospirosis in dogs, and that an organism resembling Leptospira has been demonstrated in sections of livers and kidneys of cattle suffering from the disease. The organism demonstrated in the fixed tissues has not been proved to be the cause, and when one considers the wide variety of clinical manifestations as well as the bizarre pathological changes which were encountered, there is just reason to suspect the presence of some other infectious agent, a possibility that cannot be excluded at this time. This possibility remains a subject for future investigation. In the meantime, there is a need of some established criteria which may be employed as a basis for future experimental work. Therefore, the results of our studies are herein reported under the heading of leptospirosis, regardless of the fact that we may be dealing with more than one disease.

No attempt has been made to review the literature on leptospirosis in dogs since this subject appears to have been sufficiently reviewed by Meyer, Stewart-Anderson and Eddie[1], Bloom[2], and Jungherr[3,4]. The last mentioned author reported 3 cases of leptospirosis in cattle[4] and cited four authors who appear to have recognized this as a disease entity in cattle in Russia.

HISTORY OF OUTBREAKS

Ranch 1, Presidio and Jeff Davis Counties.—Sporadic losses began on this ranch in June, 1942, and continued until the last of August, at which time 8 animals were known to be sick out of a herd of about 3,300 head of cattle of all ages. Between the last of August and September 9, there was a marked increase in the number of sick animals and by the latter part of September, at least 20 per cent of 1,700 head of cows and calves in two pastures were showing slight to well-marked evidence of illness. The disease remained confined to the cattle in two, flatland pastures and did not spread to the animals maintained in the adjoining mountainous pastures on this ranch. The incidence of the disease reached a peak about the last of September and remained constant until about October 8 with few, if any, new cases appearing after the latter part of October. During the peak of the incidence, there was an estimated morbidity of 40 per cent of the 1,700 head in two pastures with a ratio of about 3 calves to 1 grown animal. The death rate reached a peak shortly after the morbidity peak and continued into the first weeks of November with a total loss of 150 head. There was a storm of abortions, which reached a peak in the latter part of October and continued into December. The extent of this loss was not fully appreciated until 1943, when a 63 per cent calf crop was branded from the entire herd of 1,700 head of cows, compared with a 90 per cent crop in 1942 and a 91 per cent crop in 1944. An interesting feature in connection with the

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*Resigned Feb. 1, 1946.

abortions was the shipping of 250 pregnant heifers on Sept. 5, 1942, to Mexico. Between October 6 and October 20, at least 40 of these animals aborted, a condition that did not occur among the cattle that had been maintained on the ranch in Mexico. The owner estimated the loss at $35,000 which does not appear excessive in view of the actual loss in addition to the decreased sales value of the calf crop and the quality of the cattle involved. There has been no recurrence of the disease on this ranch.

Ranch 2, Brewster County.—The beginning and termination of a bad outbreak of the disease on this ranch in 1942 coincided with that of the preceding ranch. There was a loss of 80 head out of a herd of 1,360 cattle of all ages, and a storm of abortions which subsided in December. The two ranches are separated by about 30 airline miles. Sporadic losses have continued on this ranch to the time of this writing, and, although the cause of the loss in 1943 was never ascertained due to the fact that the carcasses were always found too late for an autopsy, the disease was again diagnosed in 1944 and was evidently responsible for a considerable loss. The owner has a recorded loss of 249 head of cows and calves between June, 1942, and March, 1945, and has estimated the abortions at 350 during the same period. The mortality was about equally divided between calves and grown animals. The relatively high proportion of grown animals is probably due to Senecio poisoning, which has been frequently encountered in the autopsies conducted on this ranch. Losses have occurred in every pasture on this ranch.

Ranch 3, Brewster County.—Located about 20 airline miles from the preceding ranch and 35 miles from ranch 1, the loss began on this ranch in July and terminated in December, 1942. The morbidity amounted to 28 head out of a herd of 700 cows and calves in one pasture, with no loss in the remaining pastures on this ranch. There was some abortion in one pasture, but the extent of the loss was never determined. There appears to have been no recurrence of the disease on this ranch.

Substation 9, Texas Agricultural Experiment Station, Balmorhea.—A group of 130 bulls ranging from 6 to 14 months of age were assembled at the station from 6 different ranches in Brewster, Presidio, and Jeff Davis counties, on Dec. 6, 1944, divided into 6 lots and placed on feed. The lots were established according to the classification of the bulls rather than ownership. One bull died December 15 but no autopsy was held; a second animal developed a slight illness on Jan. 13, 1945, but quickly recovered. More serious illness developed in 3 more animals between January 27 and February 2, for which an erroneous diagnosis was made. The nature of the disease was recognized on February 5 when another sick animal was found voiding red urine. Between January 27 and February 12, there was a total of 18 recognized cases in three lots, 2 having shown hemoglobinuria and 2 developed into typical chronic cases, with 1 of the latter being killed in a critical condition for autopsy. The remainder were probably all light cases, although the outcome may have been favorably influenced by the administration of sulfanilamide, a treatment that could not be adequately controlled due to the owner's desire to treat all animals following the apparent beneficial results on the first 2 cases in which it was employed.

There were no new cases between February 12 and April 2. On the latter date 1 animal developed a nasal discharge and a morning temperature of 103.8 F. The next day this animal was placed in a truck with 15 other bulls and hauled to the owner's ranch. On April 6, this animal was sick and showing a temperature of 104.6 F. Three of the 15 bulls developed an illness with high temperatures on April 12 and a fourth on April 13; the remainder showed no evidence of having contracted the disease. These animals were all treated with sulfanilamide and quickly recovered. This outbreak supplied much valuable information on the early clinical manifestations of the disease and would probably have supplied more if it had been possible to persuade the owners to allow every other sick animal to remain untreated as a control.

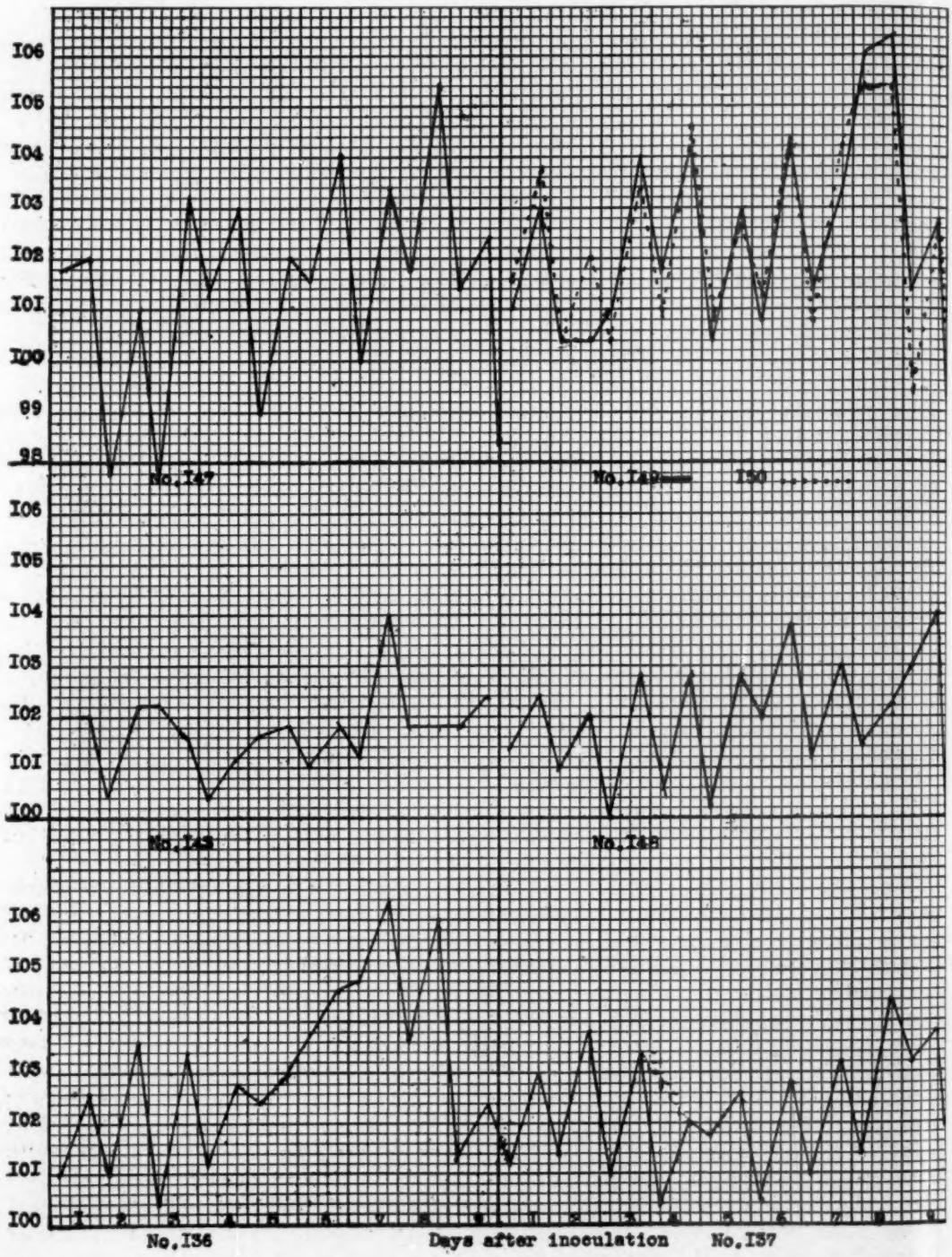

Fig. 1—Morning and afternoon temperatures of 7 cattle from the first day following inoculation to the termination of the febrile reaction.

Sporadic outbreaks were observed on two other ranches in 1942, each with a loss of 1 animal upon which autopsy was obtained, but with indications that several additional animals had passed through a minor illness. In 1944 and 1945, minor outbreaks were observed on five additional ranches, all of which had a history of one to a few abortions, but the loss from this source was not sufficient to cause any concern.

duration, poor appetite, and a marked loss of weight. Some of these cases made a quick recovery, others remained unthrifty, in spite of a good appetite, for weeks or even months but eventually recovered. This and the mildest form represented about 75 per cent of the total morbidity. In contrast to the mild forms, the acute fatal form was characterized by hemoglobinemia and hemoglobinuria from

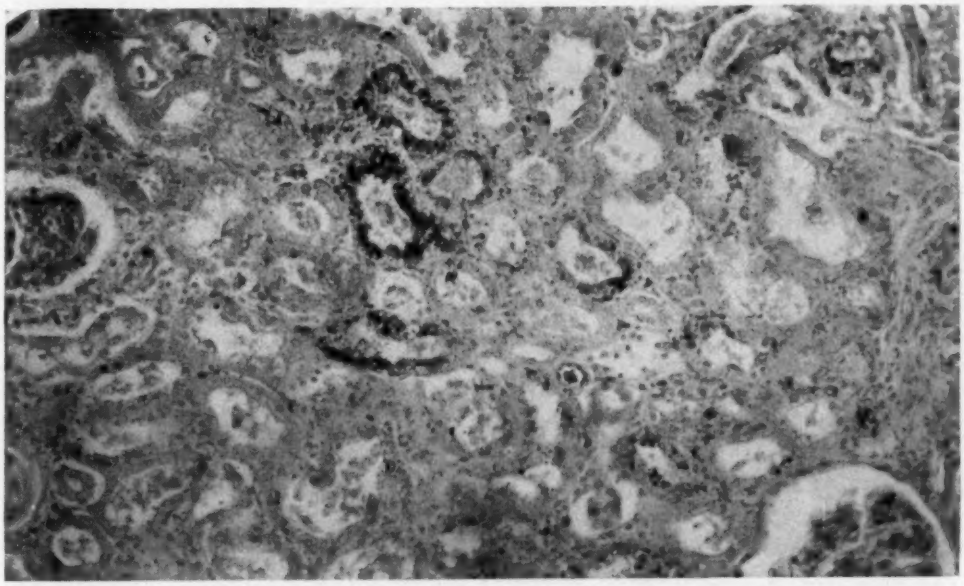

Fig. 2—Kidney of an acute fatal case, four tubules loaded with a mixture of intracellular structures and hemoglobin droplets. Weigert's fibrin stain. x 200.

Symptoms.—Temperatures ranging from 104.0 to 107.0 F., and a nasal discharge which spread over the muzzle were the first manifestations of the disease. The elevated temperature may precede the nasal discharge. At first, the discharge was a thin mucus that became mucopurulent within a few hours while the muzzle became dry and scaly. During the febrile stage there was rapid respiration, and partial loss of appetite; diarrhea may or may not have been present. From this early clinical picture there was a rapid change to recovery, or to one of three different forms of the disease. In the mildest of the three forms the fever persisted for twenty-four to forty-eight hours and was generally associated with a diarrhea of one to six days the onset. The fatal termination required from forty-eight to seventy-two hours. This appeared to be a highly fatal form, but spontaneous recoveries were observed. Between the mild and acute fatal forms there was a relatively large group of chronic cases, some of which showed a transitory hemoglobinuria but the majority did not. In this group, the diarrhea of the early stages remained constant for weeks or until death. The termination of the diarrhea was frequently marked by constipation with much mucus being expelled with the inspissated feces. In the early stages, swaying of the hind quarters while walking indicated a muscular weakness. In the majority of cases, the early loss of appetite was quickly regained but emaciation gradu-

ally developed in spite of adequate consumption of feed. Complete loss of appetite generally indicated a fatal termination within a few days, and was generally associated with a partial plugging of the nostrils with a mucopurulent discharge, and a purulent discharge adhering to the internal canthus. In many of the chronic cases, the muzzle was encrusted with a brown to brownish black scab for indefinite periods.

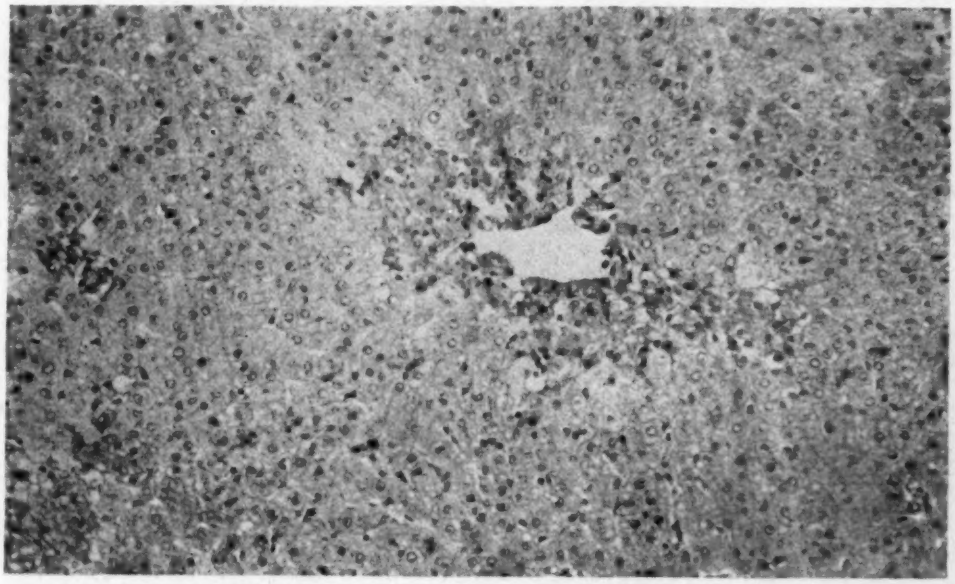

Fig. 3—Liver of an acute fatal case, focal necrosis around a central vein. Hematoxylin and eosin stain. x 150.

Sloughing of the scab left a red surface that soon changed to a light tan due to an abnormal pigmentation of the muzzle. In many cases, there was a yellow discoloration of the hair and skin, especially prominent in the white portions, which was due to an exudate which clung to the hair and surface but did not discolor the skin. Frequent urination with dribbling of urine was generally observed.

Abortion represented a mild form of the disease since in most cases there were no serious after-effects following the expulsion of the fetus. However, the typical chronic manifestations have been observed following the act of abortion. Other known causes of abortion have been eliminated in many of these cases, thus permitting no other conclusion but that this was part of the clinical evidence.

Gross Pathology.—The acute fatal form of the disease was characterized by a yellow appearance of the fat which gave the carcass of the Hereford the appearance of that of the Jersey. The color in these cases was golden rather than the greenish yellow of icterus, a condition that had not been observed. The liver, when first removed from the abdominal cavity, had a faint yellow color which developed into a definite orange-yellow after a few minutes exposure to air or light. In some cases, small necrotic foci were detected with the unaided eye. In others, there was nothing to be observed but the abnormal color. Generally, the contents of the gall bladder were moderate in amount but of a jam consistency and varied in color from golden yellow to a dark green. The kidneys varied in color from normal to a light mahogany and the cortex generally contained what appeared to be petechiæ. However, microscopic examinations indicated that the red specks were probably hemoglobin casts rather than hemorrhages, a change rarely found in the kidney. In the abomasum, numerous

small hemorrhages or ulcerations which were evidently preceded by hemorrhage in the mucosa were a constant finding. The spleen and lymph glands may or may not have been enlarged. Pneumonia was observed in 2 cases. One case had a blue urine due to a precipitate which turned green when exposed to the air, leaving the supernatant fluid red, and the preputial hairs of another case were encrusted with a blue precipitate indicating an earlier pigmented urine which was not observed at the time of the autopsy.

In the chronic form of the disease, the kidneys, abomasum, and serous surfaces presented a consistent pathological picture, but with these exceptions the gross changes were subject to a wide variation. The serous surfaces between the ribs, on the anterior and posterior surface of the diaphragm, on the spleen and sometimes the mesentery, were springled with numerous, discrete granulations which had the appearance of organized hemorrhages or fibrin.

The kidneys were generally enlarged, gray to light brown, and the cortex was sprinkled with numerous gray foci which varied in size from a few millimeters to as much as 2 cm. in diameter. The liver was generally lighter in color than normal but showed no definite lesions. Generally, the hepatic lymph glands were enlarged, edematous, and perhaps slate colored. The abomasum was either ulcerated or showed numerous areas in which healing had recently occurred; the ulcers may have been discrete circles or extensive erosions. Irregular findings consisted of a chalky white appearance of the gastrointestinal mucosa from the mouth to the duodenum in 1 case, and a similar condition confined to the mucosa of the duodenum in 2 others which presented an appearance similar to a phenol burn; atrophy of the spleen, necrosis and ulceration of the mucosa of the esophagus, a thickening of the mucosa of the jejunum and ileum, ulceration of the colon, a black pigmentation of the medulla of the kidney and associated lymph node, some degeneration of the myocardium, ulceration of the tongue, and pneumonia.

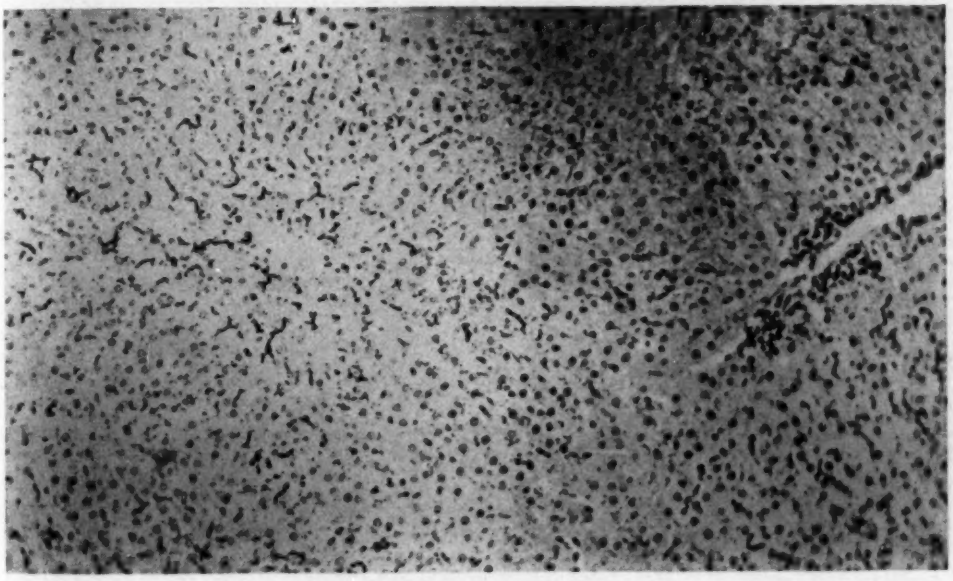

Fig. 4—Liver of same case as figure 3, filaments of static bile around an area of focal necrosis. Warthin-Starry silver method. x 150.

Microscopic Pathology.—In the liver of the acute fatal form, focal necrosis was a constant finding. This change began with a marked increase in the eosin staining

property of the cytoplasm and moderate pycnosis followed by a gradual loss of staining property of the nucleus and later with a gradual disintegration of the cell. The necrosis was generally located around the central vein in a more or less circular arrangement but was sometimes linear in outline; a stasis of bile occurred in the capillaries immediately surrounding these lesions. A prominent feature was the lack of any cellular reaction around the necrotic foci. Between the necrotic area and the periphery of the lobule there were marked albuminous degeneration and some fatty change, but the latter was not a prominent feature.

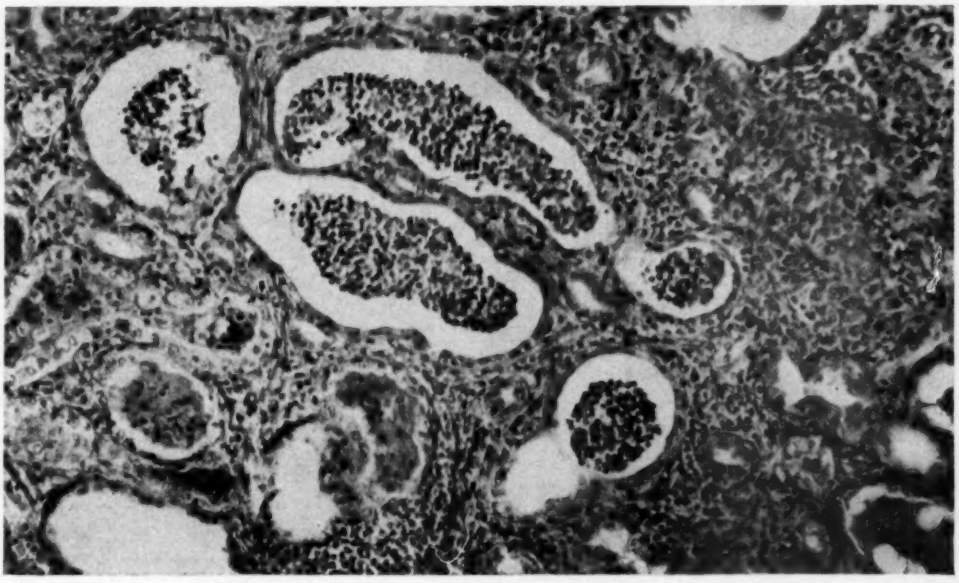

Fig. 5—Kidney of an early chronic case, pus in tubules, interstitial round cell infiltration, cellular detritus casts and regeneration of epithelium. Hematoxylin and eosin stain. x 150.

In the kidney, extensive albuminous degeneration, simple necrosis and pigmentation of the epithelium were the essential lesions. The pigment was of two types: one gave an iron reaction, the other did not. Numerous hemoglobin and albumin casts filled the tubules as well as Bowman's capsules. A prominent feature was the presence of numerous small, spherical to oval bodies in the epithelium of the convoluted portions of the tubules. They varied in size from about 0.5 to 5.0 μ in diameter and varied in numbers from 1 to as many as 30 to the cell. They stained light pink with eosin but due to the retrograde changes they were not easily detected with the ordinary H. and E. stain. With Weigert's fibrin stain they presented an excellent contrast but could not be differentiated from the blue of hemoglobin droplets which frequently occurred in the same cell. With Dunn and Thompson's[5] method of staining for hemoglobin, they stained either light yellow or light pink, which readily differentiated these structures from hemoglobin droplets which stained various shades of green. They were found only in the cytoplasm and in conjunction with pigment deposits within the same cell. Pigment deposits were also found in the epithelium of tubules in which these bodies did not occur.

The spleen showed congestion and marked hemosiderosis. In the abomasum, hemorrhage in the mucosa followed by necrosis and sloughing of the hemorrhagic area produced the ulceration which was a constant finding. There was no histologic explanation for the cause of the hemorrhage.

In the chronic form, the kidney presented

the most characteristic changes subject to considerable variation, depending upon the duration of the disease. In cases of two to three weeks' standing, there was an advanced parenchymatous and beginning interstitial nephritis. The destruction of the epithelium was more advanced than in the acute form, the lumina of many of the tubules showing a network of shredded cytoplasm; hyaline and cellular detritus

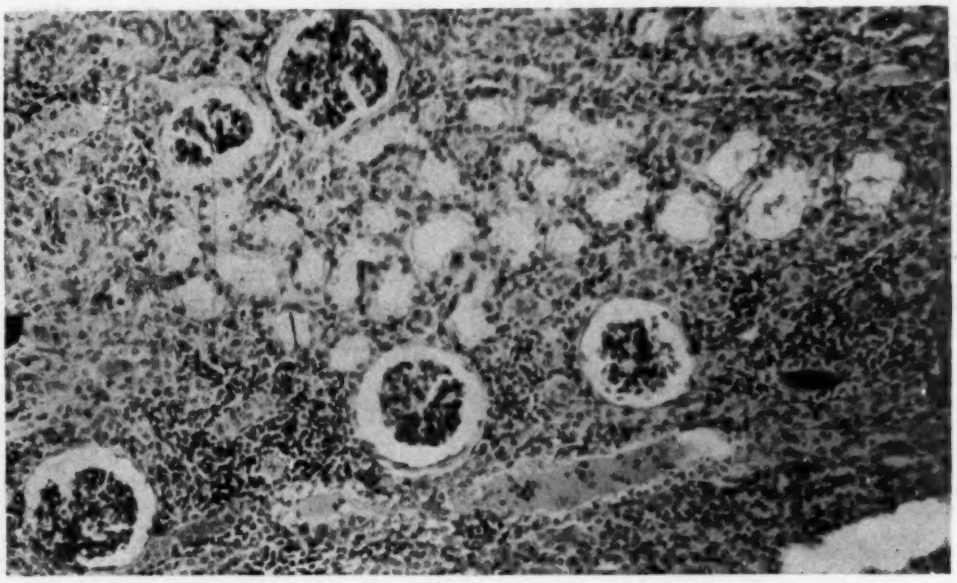

Fig. 6—Kidney of a slightly more advanced case than figure 5, cast formation, interstitial round cell infiltration and early thickening of Bowman's capsule. Hematoxylin and eosin stain. x 150.

casts were numerous and many of the tubules were distended with wandering leucocytes, of which the polymorphonuclear was the predominant type. Early fibroblastic proliferation and leucocytic infiltration appeared in the supporting connective tissue about this time. In cases of longer standing, six weeks to two months, there was marked thickening of the basement membrane of Bowman's capsule and definite cirrhosis of both the cortex and medulla. There was an extensive leucocytic infiltration which had a patchy rather than a diffuse distribution, the predominant cell being the lymphocytic type; pus cells were rarely observed. In 1 case of six months' duration, abscess formation was found, probably due to a secondary infection. Re-

generation of the epithelium formed a prominent part of the picture and explained the unexpected recovery of many grave cases, upon which an unfavorable prognosis had been made on the basis of previous autopsies on similar cases. Extensive pigment deposits were observed in the epithelium, of which the greater part gave an iron reaction with the proper stain. The structures in the epithelium of the convoluted tubules, previously described, were not as numerous but they attained greater size than in the acute form. Some were nearly as large as the containing cell and in such cases they frequently showed a pink halo around a poorly defined brown staining center with Dunn and Thompson's method. These structures were found in the epithelium of the collecting tubules of the medulla in 1 case and were also found in the chorionic epithelium of the 1 pregnant cow at the autopsy. They were associated with vacuolation of the chorionic epithelium and serous exudation between the fetal and maternal attachments.

In some instances, the liver of the chronic case showed discrete areas of focal necrosis but this was a rare finding as was also the

presence of small foci of closely packed wandering leucocytes surrounded by normal or but slightly damaged liver cells, indicating that the animal had probably recovered from the acute form and had developed into a chronic case. The usual picture was one of moderate albuminous and slight fatty change with no extensive damage to the liver in the chronic case.

hydrops and interstitial edema in some cases.

The mucosa of the abomasum showed the usual inflammatory reactions associated with ulceration and active regeneration.

The lungs, with the exception of the cases in which pneumonia was observed, adrenals and pancreas appeared to have escaped destructive action.

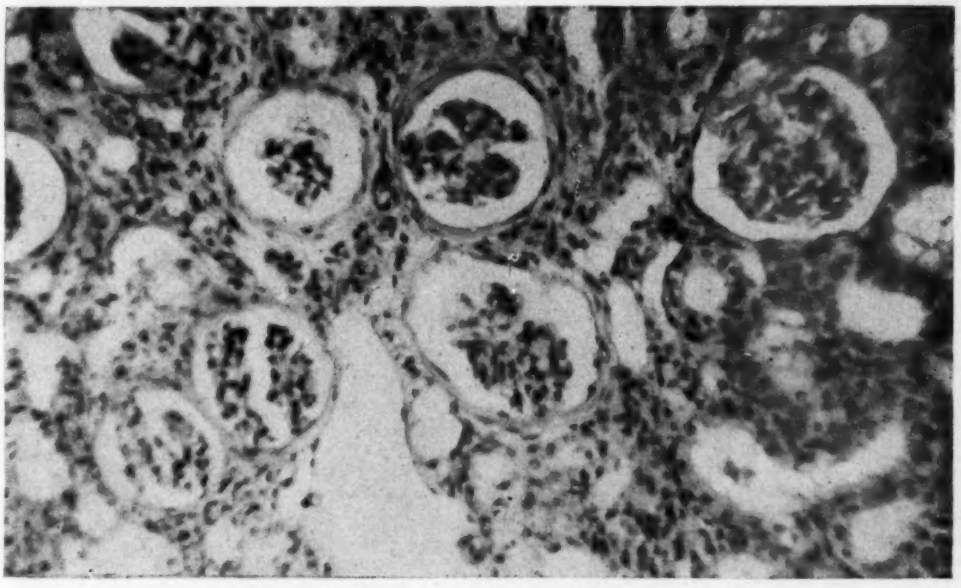

Fig. 7—Kidney of a guinea pig twenty-four days after inoculation, beginning cirrhosis and slight interstitial cell infiltration. Hematoxylin and eosin stain. x 250.

The spleen showed marked hemosiderosis and lymphoid exhaustion, the extent of the latter change depending on the length of time the animal had been affected. Two cases were found in which there had been a necrosis of the capsule and supporting trabeculae, with calcium deposits in the necrotic areas.

The lymph glands showed edema, lymphoid exhaustion, on a more moderate scale than in the spleen, and in some cases hemosiderosis.

The thickened mucosa of the small intestine observed at the autopsy was found to be due to a lymphoid hyperplasia of such an extent that a tube of lymphoid tissue was formed between the mucosa and the muscular wall. The myocardium showed

BLOOD STUDIES

Two affected calves were picked up on the range and brought to the laboratory for observation. Both were showing a diarrhea at the time but from their general appearance it was evident that they had not been sick more than two or three days. There was no evidence of hemoglobinuria during the observation period on these animals. The diarrhea persisted in both calves and there was a rapid loss of weight in spite of a good appetite and the consumption of large amounts of alfalfa hay. Both were killed for autopsy in an emaciated condition, 1 five weeks and the other ten weeks after being placed under observation. Both developed a more or less typical, chronic type of the disease. One animal had

a red blood cell count of 6.5 million per cubic millimeter of blood (about 2 million below normal minimum for this region) when first placed under observation and five weeks later the count had dropped to 4.8 million. The leucocytic count was 12,900 on the first day and 16,100 on the day of autopsy five weeks later. The second animal had a red blood cell count of 9.5 million

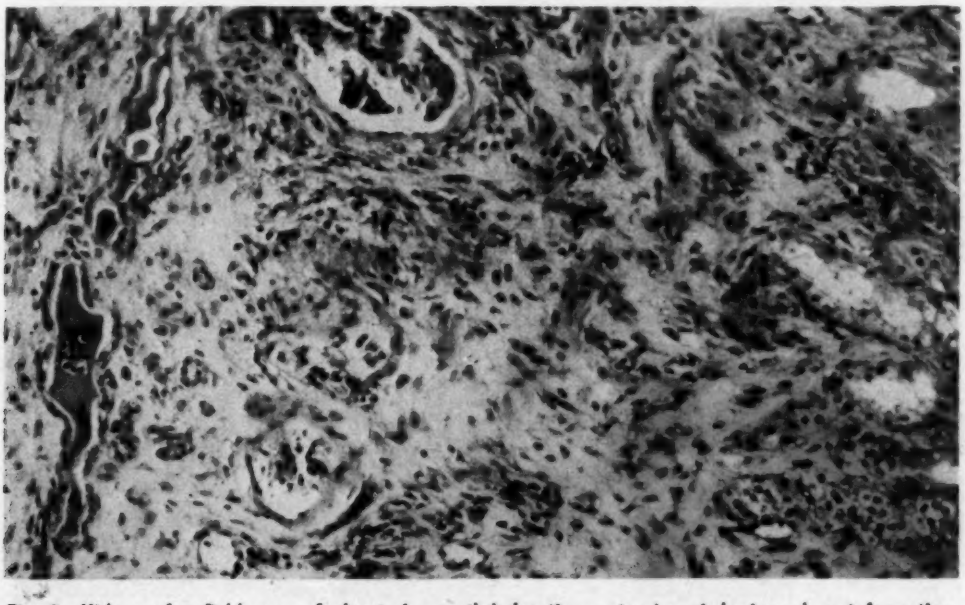

Fig. 8—Kidney of a field case of about six months' duration, extensive cirrhosis and cast formation. Hematoxylin and eosin stain. x 250.

per cubic millimeter of blood on the first day and 4.3 million ten weeks later when it was killed for autopsy. The first leucocytic count on this animal showed 12,200 leucocytes per cubic millimeter of blood, which increased to 23,000 and then gradually dropped to 13,800. The average leucocytic ratio for both animals was: polymorphonuclear, 17; lymphocytes, 61; mononuculear, 9; eosinophiles, 1; and megaloblasts, 12. The development of anemia in both animals was evidence of a blood dyscrasia without hemoglobinuria in chronic cases, which was in keeping with field observations, as most chronic cases have disclosed an anemic condition upon autopsy. No change was observed in the blood of the light cases which were produced as a result of inoculation.

ANIMAL INOCULATIONS

From the first investigations, the presence of an infectious agent was suspected and attempts to reproduce the disease by animal inoculation were made. One calf, 2 yearlings, and a 3-year-old cow were given subcutaneous injections of an emulsion of liver, kidney, and spleen from an acute case of the disease. In addition, the calf was given an intravenous injection of blood and 1 of the yearlings was fed a pound of ground liver and kidney. These animals were kept in a corral with the 2 field cases above described for the full period during which these 2 animals were under observation. The temperatures of the 4 experimental animals remained normal for a period of twenty-one days and there was no evidence of a transmission of the disease. With this failure in mind, our attention was directed towards a search for unidentified, poisonous plants and much needless work was conducted before this theory was discarded, and animal inoculations again attempted.

On Sept. 7, 1944, 2 calves (136 and 137) were injected both subcutaneously and in-

travenously with an emulsion of kidney and blood which had been obtained from an acute case. The same emulsion, to which was added 150 cc. of urine, was administered to a third calf *per os.* This calf has remained healthy to the time this was written.* Six days after inoculation, calf 136 developed a nasal discharge and a fever (fig. 1) which lasted for forty-eight hours, the nose became dry and scaly and peeled shortly after the fever subsided. It developed a diarrhea on the seventh day which continued for three days and then subsided. It remained in an unthrifty condition for about three months but is apparently healthy at this time. Calf 137 had a temperature of 104.4 F. the evening of the eighth day, a slight nasal discharge, and a dry nose. The next morning these symptoms had disappeared and a diarrhea had developed from which it recovered on the following day. This calf appeared healthy forty-eight hours after the one high temperature and has remained so to the time of this writing.*

On Feb. 6, 1945, blood was collected from a young bull which was showing a temperature of 105.0 F., a hemoglobinemia and hemoglobinuria. The blood was defibrinated and injected in 10-cc. amounts, both subcutaneously and intravenously into 2 calves and 4, 2-year-old heifers. In addition, a few drops were instilled in the eyes of each animal. The temperature reaction of heifer 143 is given in figure 1. There was no evidence of a transmission of the disease in the other 5 animals. Seven days after inoculation, heifer 143 had a temperature of 104.0 F. at 5 p.m., and a slight nasal discharge. The next morning the temperature was normal, the nasal discharge had ceased, and there was no evidence of after-effects from this short period of illness.

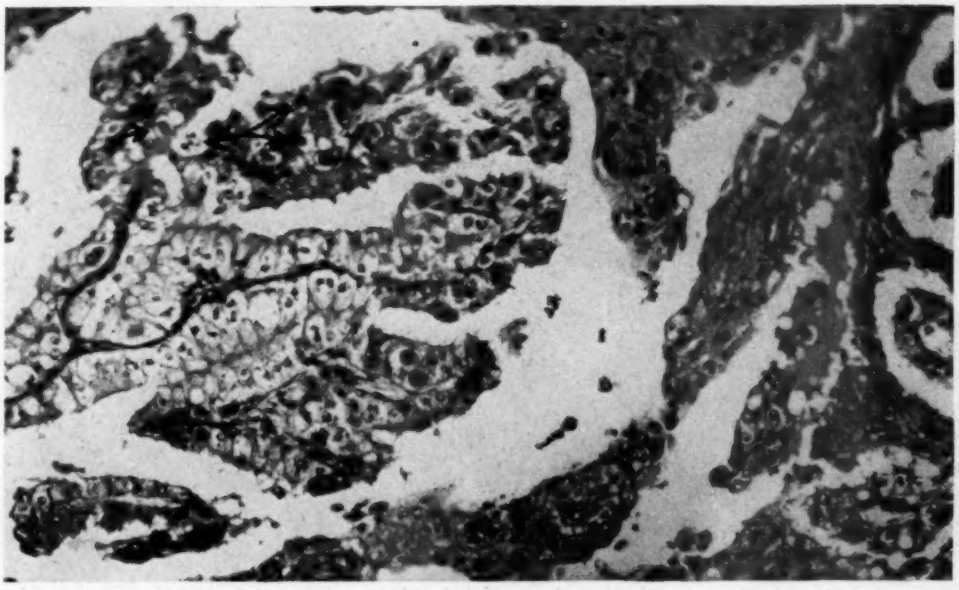

Fig. 9—Chorionic villus, same as figure 10, edema separating the fetal and maternal placenta, degeneration of the chorionic epithelium, three attached arrows pointing to intracellular structures with brown staining centers, one arrow pointing to a larger structure with no brown center. The pink halo around the brown centers is very faint. Dunn and Thompson's method. x 150.

The results of the foregoing inoculations suggested the presence of immunity in the small herd of Hereford cattle maintained by the laboratory for experimental work. Therefore, 7 Jersey calves which had been reared close to the city limits several miles distant were purchased for further inoculation experiments. On Feb. 16, 1945, a young bull was found at Substation No. 9, near Balmorhea, with a temperature of 106.2 F.,

*June, 1945.

and a nasal discharge at 9 a.m. This animal appeared healthy the day before. The evening of Feb. 16, the temperature of this animal was normal and the next day there was nothing to indicate that it had passed through a fleeting period of illness. Blood was collected from this animal at 9 a.m. on Feb. 16, defibrinated and injected into 4 of the Jersey calves (147, 148, 149, and 150), 2, 2-year-old heifers and 3 cows from the laboratory herd. Each animal received 10 cc. intravenously and 5 cc. subcutaneously, and an instillation of a few drops in the eye. The other 3 Jersey calves were left as controls and carried normal temperatures for a period of three weeks. There was no evidence of illness in the 2 heifers and 3 cows, thus further demonstrating the presence of immunity in the laboratory herd. The temperature charts for the 4 Jersey calves are given in figure 1. All 4 animals developed a temperature reaction as a result of the inoculation, and a nasal discharge; in addition, calf 150 developed a diarrhea from which it quickly recovered with very little after-effects. It is of interest to note that calf 148 which showed the least temperature reaction developed the only serious after-effects observed in the 4 animals. This animal developed a diarrhea on the tenth day, which lasted for one week, and lost considerable weight. It was unthrifty for one month after which rapid recovery occurred. The temperature charts for calves 147, 149, and 150 show some variation for which no explanation is available. Calf 147 showed subnormal temperatures on the mornings of the second and third day. Calves 149 and 150 showed nearly identical temperature reactions and at variance with those of the other animals. Both showed evening temperatures of 104 F. or better on the third and fourth days. As the temperatures were accompanied by no other evidence of illness, they probably have no significance. On the other hand, the temperatures of 104.2 F. on the evening of the sixth day are significant, since a nasal discharge appeared during the following night and was accompanied by a partial loss of appetite. The drop to normal temperature on the seventh and a second rise on the eighth day is another feature for which no explanation is offered.

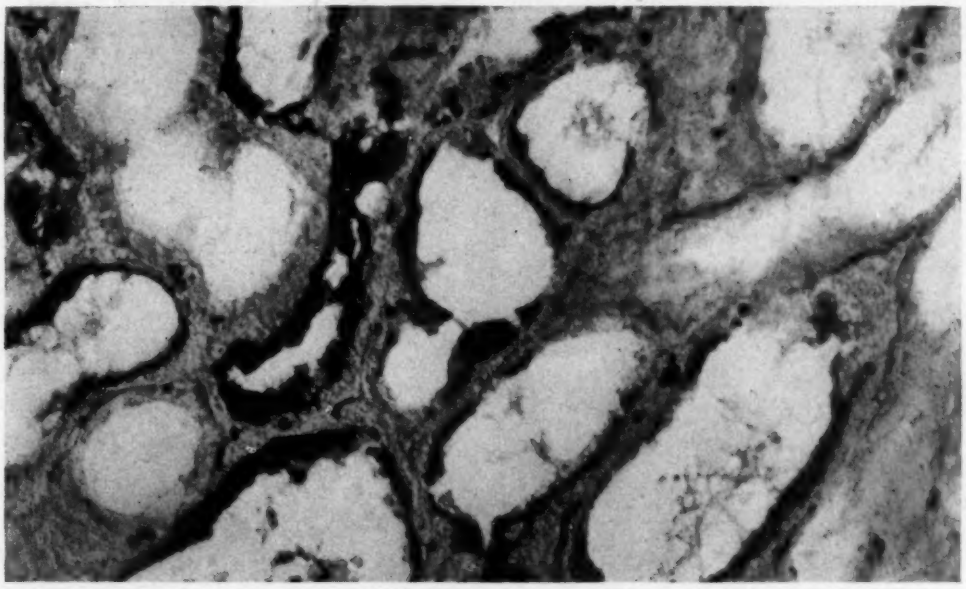

Fig. 10—Kidney of a chronic case, masses of leptospira adhering to the epithelium within the lumina of several tubules. Warthin-Starry silver method. x 350.

A summary of cattle inoculations shows that a febrile disease was produced in 7

out of 20 inoculated animals with negative results being obtained with *per os* administration in the case of 2 animals.

Blood from a field case which had a temperature of 104.6 F. at the time the blood was collected was injected intraperitoneally in 2-cc. amounts into 3 rabbits and 6 guinea pigs. The rabbits have shown no ill effects as a result of the inoculations but 2 of the 6 guinea pigs began to lose weight shortly thereafter without a febrile reaction and were killed for autopsy. One guinea pig was killed on the seventh day following inoculation and as nothing of significance was found upon autopsy, its poor physical condition was considered to be due to extraneous causes. The second guinea pig was killed twenty-four days after the inoculation and upon autopsy gray, atrophic kidneys were found which had a wrinkled appearance. Microscopically, the lesions in the kidneys of this case were identical to those found in the kidneys of chronic cases in cattle.†

Three sheep and 4 goats were inoculated with blood from a field case with entirely negative results.

STAINING FOR LEPTOSPIRA

The use of several of the aniline dyes failed to demonstrate the presence of an organism in smears and tissues obtained from autopsies; therefore, the use of Levaditi's and the modification of this method, as described by Meyer, *et al.*, for staining of spirochetes was employed. For this purpose, 3 acute and 3 chronic cases were selected with blocks of liver, kidney, and spleen from each autopsy being stained by both methods. After prolonged search, a spiral organism was never found in sections from any of these blocks. Incidentally, the Warthin-Starry method for the staining of spirochetes as described by Kerr[6] has failed to demonstrate the presence of Leptospira in the tissues of the 3 acute cases, very few organisms in the kidneys of 2 of the chronic cases, and moderate numbers in the kidneys of the third chronic case. The latter method has proved satisfactory for staining the organisms in tis-

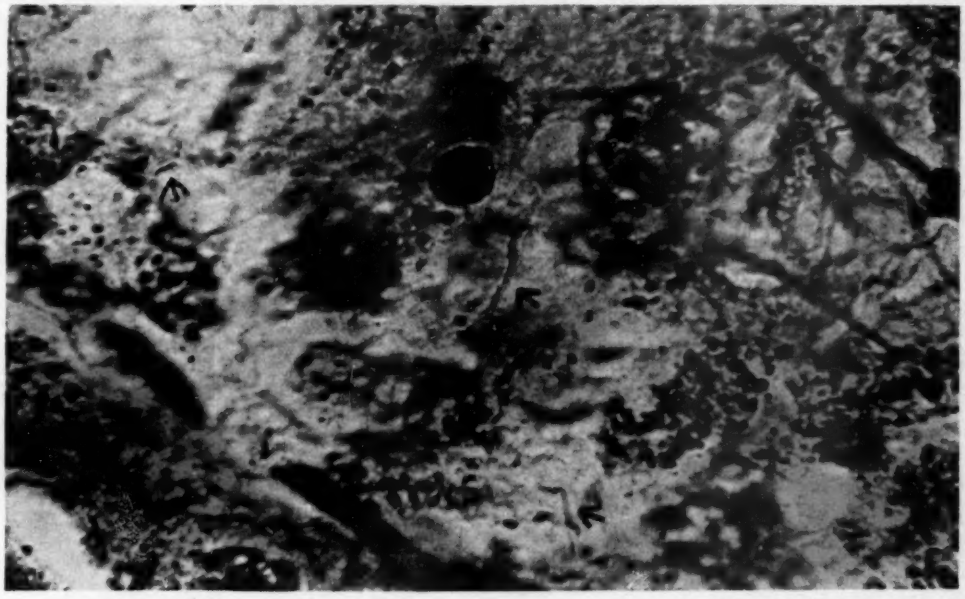

Fig. 11—Leptospira in a chorionic villus, one complete organism in focus, several with part of the organism in focus, same as figure 9. Warthin-Starry silver method. x 1285.

†The disease was transmitted from guinea pig to guinea pig through three series of experiments, using emulsions of kidneys. Leptospira were never demonstrated in the diseased kidneys.

sues but only a small percentage of the organisms presented the "hook" which is considered characteristic for other Leptospira. Furthermore, the borders of the organisms presented a wavy outline but an actual spiral could not be detected. Sections from livers, kidneys, and spleens of 17 out of 20 autopsies have been stained by the Warthin-Starry method and this type of organism was found in large numbers in the kidneys of 6 and the livers of 2 cases. An occasional organism was found in the kidneys of 8 and the livers of 4 cases, but no organisms were found in the kidneys of 3 and the livers of 11 cases. The organism was found in the chorionic villi of the 1 pregnant animal which had been subjected to autopsy. It was never found in the spleen, lymph glands, nor in hemorrhagic and ulcerated areas of the abomasum. In the kidneys, the organisms were found adhering to the borders of the epithelium within the lumina of the tubules or protruding into the epithelium to the depth of a few microns. They were never found in the blood vessels. The same is true for the liver and chorionic villi. The organism was not found in the kidney, liver, and spleen of the guinea pig which presented the characteristic, chronic lesions in the kidney.

AGGLUTINATION TESTS

The serum from 6 of the experimental animals and 3 field cases was tested with the rapid plate agglutination test with both *Leptospira icterohemorrhagiae* and *Leptospira canicola* stained antigens.* No agglutination was obtained in either of the two antigens from the serum of any of the cattle in dilutions of 1 to 2 and 1 to 1000.

TREATMENT

When the outbreak at Substation 9 was diagnosed and it was obvious that some sort of treatment should be attempted, it was planned to treat every other case with sulfanilamide, leaving the remainder as controls. However, the results with the first 2 treated animals appeared so satisfactory that the owners were reluctant to permit the retention of proper controls. On that account, no definite statement can be made but the results did indicate that this treatment may be of benefit during the febrile stage of the disease but that in the case of an animal sick for three or four days and the fever subsided, the use of sulfanilamide may be contraindicated.

*Antigen supplied by Lederle Laboratories, Inc., Pearl River, New York.

DISCUSSION

The mere presence of an organism does not constitute proof that it causes a disease for which the specific cause has not been established. Therefore, the significance of the Leptospira in the tissues of these animals cannot be interpreted until Koch's postulates have been satisfied. This procedure will not be attempted until the necessary equipment has been added to the laboratory.

A consideration of the disease-complex presented many unanswered questions if the etiology is attributed entirely to Leptospira and the presence of a second agent is not considered. Leptospira is indeed a versatile organism if it is capable of producing a disease with such a wide variety of clinical manifestations which ranged all the way from a transitory illness of but a few hours to death within forty-eight to seventy-two hours, or a chronic condition associated with a bizarre and, in some cases, incongruous pathology, such as a lymphoid exhaustion of the spleen and lymph nodes and a hyperplasia of the lymphatic tissue of the small intestine. Another condition which left much to the imagination was the damage to tissues entirely out of proportion to the number of organisms found if Leptospira was the primary cause. This is especially true in the liver of acute cases in which Leptospira could not be demonstrated in the presence of extensive damage. As a matter of fact, this organism was not demonstrated in any of the tissues in 3 out of 4 acute, fatal cases which were examined *post mortem*. In this connection, some of the most extensive lesions in the kidneys of chronic cases were associated with the presence of very few Leptospira, whereas moderate

lesions have been associated with massive accumulations of this organism within the tubules. In addition, the cause of the hemorrhages and ulceration of the abomasum remains unexplained as this was not primarily a disease of the blood vessels and neither Leptospira nor thrombosis were found in or around these lesions. In order to reconcile the extensive destruction of tissue to the number of organisms demonstrated, the presence of a potent toxin would have to be assumed, a contingency that does not appear to have been demonstrated elsewhere in the case of this organism.

The resemblance of the pathology to the recognized pathology of leptospirosis in dogs presented some positive evidence for the classification of the disease in cattle as leptospirosis; however, noticeable differences are to be observed. On the positive side, ulceration of the stomach of both species was the most outstanding lesion. Microscopically, the focal necrosis of the liver accompanied by no cellular reaction and the parenchymatous and interstitial nephritis are common to both dogs and cattle. However, in cattle, the interstitial nephritis was as much cirrhotic as infiltrative, whereas in dogs it appears to be predominantly infiltrative. Furthermore, the vacuolation of the renal epithelium as reported by Bloom was not observed in these cattle. The icteric and hemorrhagic forms recognized in dogs were not observed in these cattle as there was no true icterus, and the only location where hemorrhages were consistently found was in the abomasum in the acute, fatal form.

This disease appears to have added another infectious cause for abortion in cattle and is evidently a factor in the abortion problem of the Southwest, which has a history of at least twenty-five years' standing.

In some of the more recent outbreaks of this disease, a history of a few sick animals, generally calves, with a slight loss preceding the storm of abortions has been obtained. In some of these outbreaks, the now recognized infectious causes of abortion have been eliminated, thus prompting the conclusion that we are dealing with one of the mild forms of this disease. One abortion appears to confer immunity as there is no history of two or more abortions having occurred in the same individual.

In the natural spread of the disease, the urine should be considered the most important factor, but if this is the case it would be logical to expect a greater spread of the disease in the feedlot than actually occurs on the ranges. Such was not the case as a much higher morbidity was observed in large pastures than actually occurred in the feedlot. Evidently, other methods of dissemination are involved and the possibility of insect transmission must be considered. This possibility appears more significant in view of the fact that the serious outbreaks on the ranges occurred during the season for biting insects but, in the case of the bulls, the season of the year more or less eliminated this factor.

Temperatures lower than 104.0 F. were not considered as an indication of fever. This allows a greater variation for the normal than is generally recognized for cattle, but is justified from past experiences which have shown that the recognized normal temperature for the ox does not apply to the range animal not accustomed to the handling required to obtain a temperature reading.

SUMMARY

1) A febrile disease of cattle involving both epizoötic and enzoötic outbreaks is reported.

2) Four forms of the disease were recognized; first, a slight illness of but a few hours' duration; second, a continuation of the first and followed by noticeable ill effects but eventual recovery; third, an acute fatal form characterized by hemoglobinuria and focal necrosis of the liver; and fourth, a chronic form characterized by parenchymatous and interstitial nephritis and a wide variety of other lesions.

3) The mild forms of the disease were reproduced in 7 calves by injection of blood from field cases, and the chronic lesions of the kidneys were reproduced in 1 out of 6 guinea pigs. Sheep, goats, and rabbits did not appear to be susceptible.

4) The pathology of the disease has much in common with that of leptospirosis in dogs.

5) Leptospira were demonstrated in the tissues of some but could not be found in all of the field cases.

6) The serum of 6 experimental and 3 field cases did not agglutinate either *Leptospira canicola* or *Leptospira hemorrhagiae* antigens.

Leptospira has not been proved to be the etiology of this disease.

ACKNOWLEDGMENT

The writer wishes to acknowledge his appreciation to Dr. Fred Major for his continued coöperation and assistance in the field study of this disease; to Dr. W. T. Huffman for his assistance in the investigation of the first outbreak; to Dr. H. Schmidt for his suggestions and moral support; and to Mr. George Jones and Mr. Hillsman Davis for their continued coöperation.

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Observations on the Prophylactic and Curative Value of Sulfaguanidine in Swine Coccidiosis

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IN RECENT YEARS, it has been demonstrated that sulfaguanidine possesses prophylactic properties against certain species of coccidia of mammals and birds. Foster, Christensen and Habermann[1] reported that daily administration of sulfaguanidine prevented lambs from acquiring natural coccidial infections and reduced to insignificant proportions heavy, existing, subclinical, natural infections. Levine[2] and Farr and Allen[3] noted that daily administration of sulfaguanidine in poultry mash had marked inhibitory effect on experimental coccidial infections in chicks. The last two authors also observed that birds treated after coccidiosis symptoms had started were not significantly benefited, although oöcyst production was sharply reduced. Boughton[4,5] found that, in certain experimental and natural coccidial infections in calves, sulfaguanidine reduced the severity of the disease and of oöcyst discharge and protected the animals from fatal infections.

The above reports led the writers to investigate the value of sulfaguanidine in swine coccidiosis since this disease is of considerable economic importance locally and elsewhere. The purpose of this paper is to report the results of observations made in the use of this drug in young pigs experimentally infected with mixed species of swine coccidia, *Eimeria deblieckí* and *Eimeria scabra*.

MATERIALS AND METHODS

The 18 pigs used in the present work were about two to three months old, except 1 (No. 10) which was four months old. All had been raised in clean, concrete pens by the Department of Animal Husbandry of the University of Hawaii Agricultural Experiment Station.

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For each of the six experiments reported, healthy, coccidia-free pigs of the same litter were used except that pig 10 of experiment 5 was from another litter. The initial determination for presence of coccidial oöcysts was made by examining the feces by the simple smear preparation method and by Faust's[6] zinc sulfate centrifugal, flotation technique. Each pig was maintained in an individual concrete pen which was thoroughly washed daily and disinfected weekly. The feed and water for each pig were kept in small wooden boxes built in such a way that the animal could not step inside the box. The floor and sides of the water box were lined with sheet metal. Fresh feed and water were placed in the boxes each day. The feed consisted of the following ingredients: rolled barley, 62.5 per cent; cane molasses, 10 per cent; meat meal, 16 per cent; soybean oil meal, 11 per cent; and salt, 0.5 per cent.

The coccidial oöcysts used in these studies were originally recovered from mixed feces of 3 naturally infected sows from a local piggery. The oöcysts were of two species, *E. deblieckí* and *E. scabra*, the former being more abundant than the latter, in a ratio of about 3 to 1. After these oöcysts had sporulated, they were used to infect 1 pig. This process of infection was repeated in a few other pigs until the desired concentration of the oöcysts in the feces was obtained. These oöcysts were used to infect pigs in experiment 1, and were perpetuated in subsequent experiments.

The oöcysts were concentrated and cultured from the feces by emulsifying the latter in 1 per cent potassium dichromate solution and washing it consecutively through 20- 40- and 80-mesh metal sieves. The washing was collected in 1,000-cc. beakers and allowed to settle for twenty-four hours. The supernatant fluid was then siphoned off and the sediment poured into moisture dishes in sufficient amount to cover the bottom of the dish to a depth of about 8 mm. The dishes were kept uncovered at room temperature and the fluid agitated for a few minutes each day for a period of about fifteen to twenty days. Water was added to the cultures to replace that which evaporated. After this period the cultures were centrifuged and the sediment washed several times until there was no evidence of the chromate color. The sediment was then stored in a refrigerator and used for infection within two weeks. Just

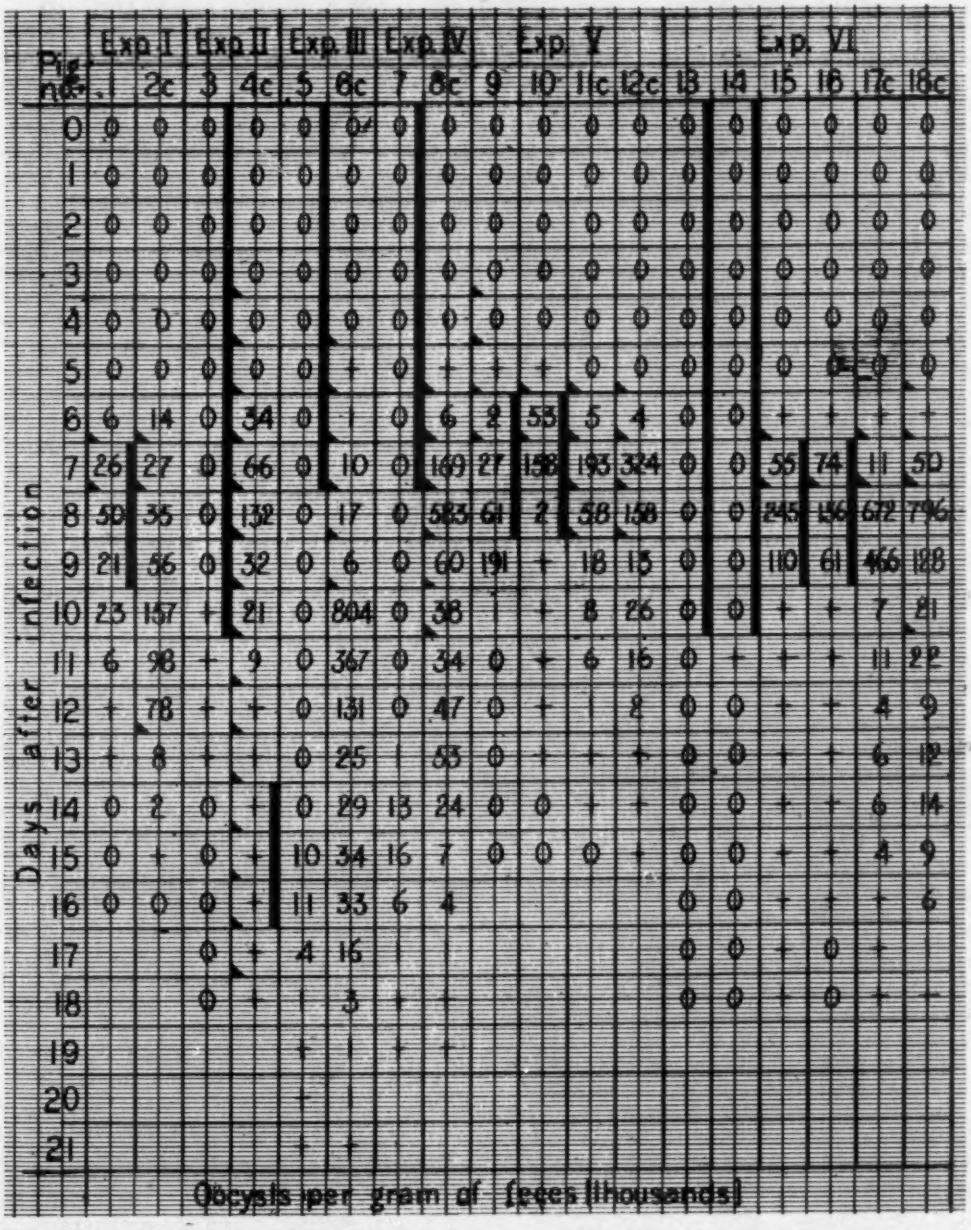

Fig. 1—Daily oöcyst counts (in thousands) made from the feces of 18 pigs following experimental infection with coccidial oöcysts. Pigs designated with the letter "c" did not receive drug treatment and served as controls. The others received sulfaguanidine treatment for the number of days designated by the solid black lines. Squares in which there are black triangular areas represent days when the feces were very soft or watery.

before the sediment was used a count was made to determine the approximate number of oöcysts for each cubic centimeter; this ordinarily amounted to about ½ million. Each of the pigs used in these experiments was fed enough culture to contain about 20 million oöcysts, except for pigs of experiment 4, which were given about 30 million oöcysts. The culture in experiments 1 and 2 (*see* fig. 1) was given on two consecutive days, mixed with small amounts of feed. In experiments 3 to 5, the culture was given with feed in one day, and in experiment 6, it was given in 1 dose with the aid of a syringe-stomach-tube outfit.

Sulfaguanidine was administered to each pig by mixing it with a small amount of feed once each morning at the dose rate of 1 Gm. per 10 pounds of body weight. Pigs which received prophylactic treatment received the drug two days before infection, on the day of infection, and for seven or ten days more. To pigs which received curative treatment, the drug was administered for three consecutive days beginning with the second day of oöcyst production.

During the period of the experiments, daily records were kept of the condition of each animal, amount of feed consumed, consistency of the feces, and the approximate number of oöcysts discharged per gram of feces.

The method of preparing the feces for counting was similar to that reported by Swanson and Kates.[1] A 24-hour, fecal output was thoroughly mixed with a spatula and a representative 5-Gm. sample taken. This was placed in a 300-cc. Erlenmeyer flask with 200-cc. of N/10 sodium hydroxide and glass beads and stored in a refrigerator overnight. The preparation was then thoroughly shaken and dilution counts made. Each count was made by taking a 0.1-cc. sample immediately after shaking the flask and determining the number of oöcysts in the sample. This was done by a method devised by the senior author, which consisted in dropping the sample on a microscope glass slide and covering it with a thin glass slide 1 by 1.25 inches. One side of this cover slide was marked with a diamond glass cutter with thin lines subdividing it into 16 equal squares. In covering the sample, the side containing the squares was placed in contact with the fluid to be examined. With the use of a low power objective, a count was made of the number of oöcysts in four of the squares running diagonally from the upper left hand corner to the lower right hand corner. The number of oöcysts thus counted was multiplied by 4 and the resulting product was multiplied by 400. This calculation gave the approximate number of oöcysts per gram of feces. For each flask preparation, two counts were made and averaged. The result of the daily oöcyst count for each pig is summarized in figure 1. The number of oöcysts is reported in even thousands. A count of 800 oöcysts is reported as 1,000. If the number of oöcysts was below 800 per gram of feces it is reported by the plus sign. When the number of oöcysts in the feces was very low, as in the beginning or end of oöcyst discharge, it was ascertained by the use of the zinc sulfate centrifugal flotation technique.

TABLE I—Comparative Body Weights of Treated and Untreated Pigs Two Weeks after Experimental Coccidial Infection

	Treated				Untreated control			
	Pig No.	Initial weight (lb.)	Weight after 2 weeks (lb.)	Gain or loss (lb.)	Pig No.	Initial weight (lb.)	Weight after 2 weeks (lb.)	Gain or loss (lb.)
Prophylactic treatment group	3	28	[1]	satisfactory gain	4	30	[1]	body loss
	5	47	[1]	satisfactory gain	6	55	[1]	no gain
	7	67	90	23	8	65	72	7
	13	40	51	11	17	35	39	4
	14	34	46	12	18	42	44	2
Curative treatment group	1	28	[1]	slight gain	2	30	[1]	slight gain
	9	25	30	—5	11	30	30	0
	10	88	108	20	12	30	30	0
	15	38	38	0	17	35	39	4
	16	32	33	1	18	42	44	2

[1]Actual weight not recorded.

EXPERIMENTAL OBSERVATIONS

The observations reported in this paper are based on six separate experiments, the details of which are summarized in figure 1. For clearer presentation of this report, the animals used are segregated into two groups: (*A*) those in which it was intended to determine the prophylactic value of sulfaguanidine (experiments 2, 3, 4, and part of 6), and (*B*) those intended to determine the curative value of the drug (experiments 1, 5, and part of 6). The main feature in each of these two groups is the difference noted between the treated and the untreated control pigs with regard to symptoms of diarrhea, oöcyst discharge, and body growth. These items are discussed below for each group.

A) *Prophylactic Treatment.*—(1) Diarrhea: The 5 pigs (3, 5, 7, 13, 14) which received sulfaguanidine prophylactically showed no diarrhea. All the untreated con-

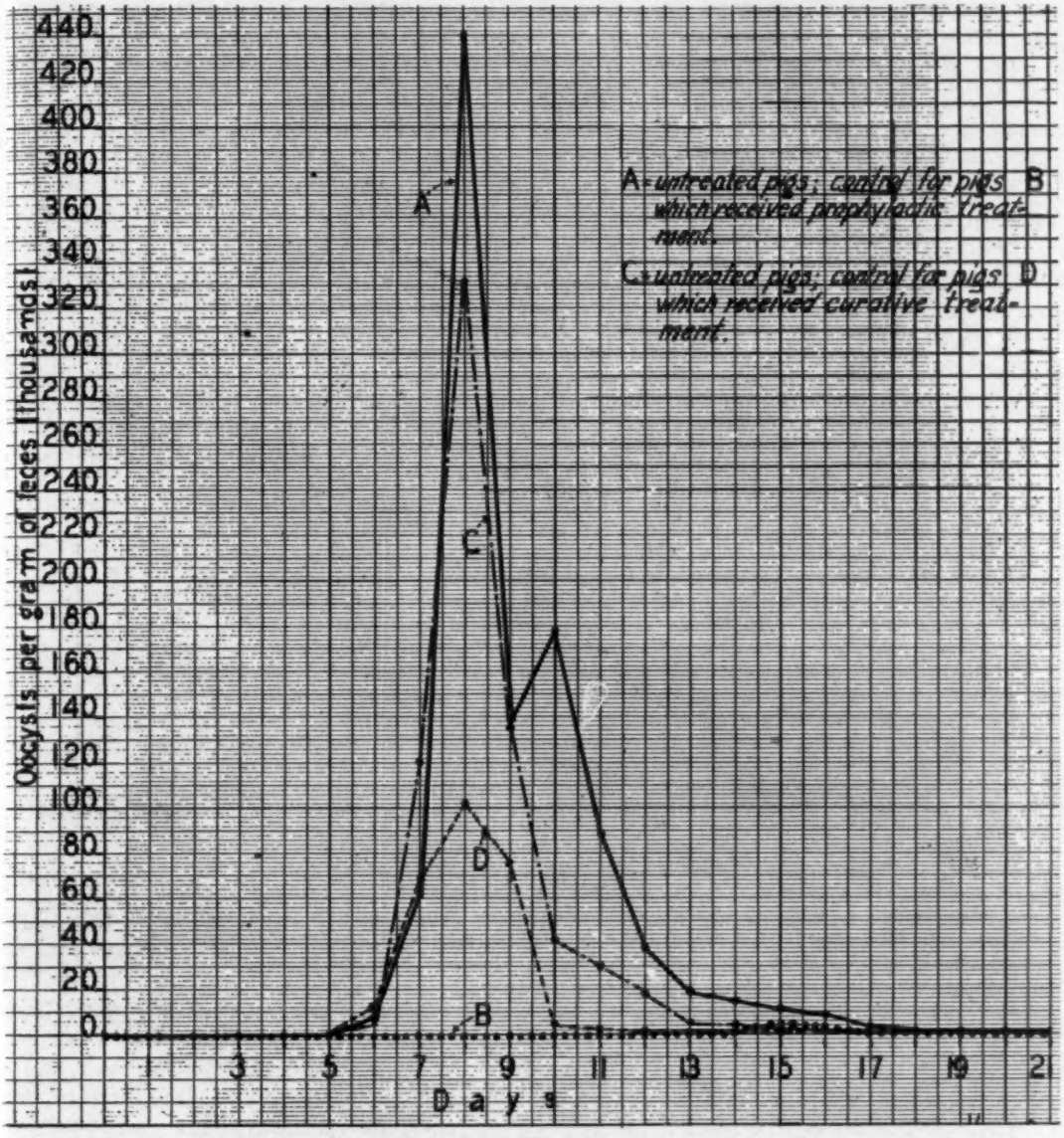

Fig. 2—Average daily oöcyst counts (taken from fig. 1) from groups of pigs which received prophylactic and curative treatment, and average daily oöcyst counts from their respective untreated controls.

trol pigs (4, 6, 8, 17, 18) developed diarrhea, which started from the third to the sixth day after experimental infection and lasted for a period of two to fifteen days (fig. 1).

2) Oöcyst discharge: A difference was noted between the pigs which received the drug for ten days and those receiving it for seven days. Of those receiving the drug for ten days, pig 3 showed a few oöcysts from the tenth to the thirteenth day after infection, pig 13 showed no coccidia, and pig 14 showed a few oöcysts on the eleventh day. These oöcysts were very few and were detected by the use of the flotation technique. Of the 2 pigs which received the drug for seven days, sufficient oöcysts were discharged by pig 5 to make dilution counts from the fifteenth to the eighteenth day after infection, and by pig 7 from the

thirteenth to the seventeenth day after infection.

In the untreated control group (4, 6, 8, 17, 18), oöcysts were found in the feces beginning with the fifth or sixth day after infection. Oöcyst discharge in sufficient number to be detected in two dilution counts lasted for a period of six to fifteen days.

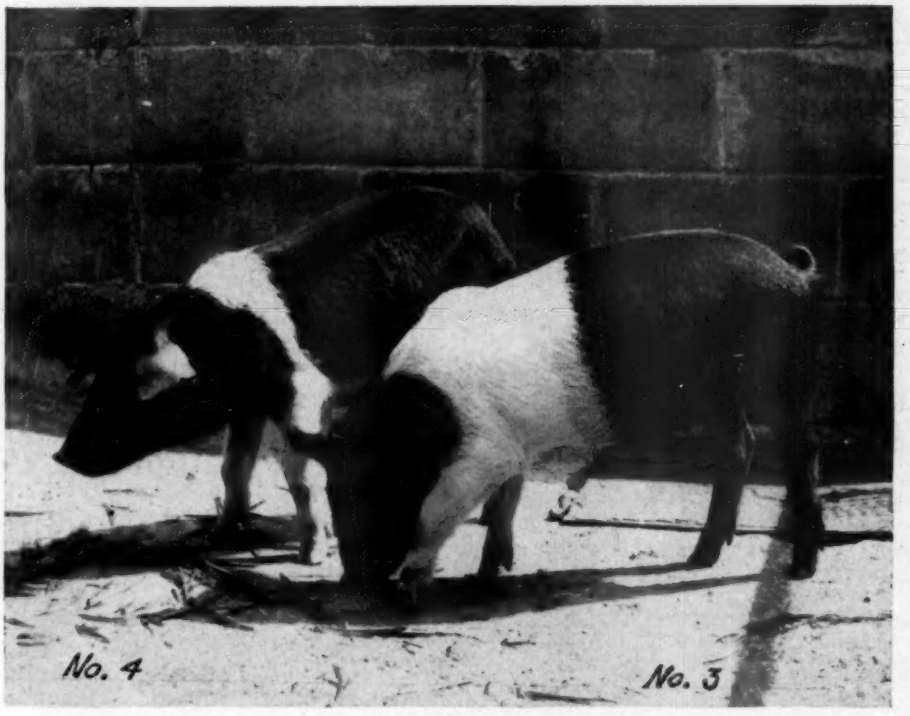

Fig. 3—Pigs 3 and 4, of the same litter, experimentally infected with coccidia. Pig 3 received sulfaguanidine therapy and developed no symptoms of coccidiosis. Pig 4 received no treatment and developed severe symptoms of coccidiosis.

The highest peak of oöcyst discharge occurred usually on the eighth day after infection. In 1 case (pig 6) this occurred on the tenth day and was associated with the production of many bizarre shaped oöcysts of *E. scabra*. There was a possibility that this represented an abnormal condition.

3) Growth: All the pigs (3, 5, 7, 13, 14) which received the drug prophylactically consumed their usual amount of feed and made normal body gain (table 1 and fig. 3). The untreated control pigs (4, 6, 8, 17, 18) made little or no body gain. The failure to grow was undoubtedly due to the presence of the parasite, which caused, among other things, extensive diarrhea and loss of appetite. The latter symptoms were observed about the fifth or sixth day of infection and continued for one to two weeks.

B) Curative Treatment.—(1) Diarrhea: Treatment in this group of pigs (1, 9, 10, 15, 16) started on the second day of oöcyst production, which occurred on the sixth or seventh day of infection. At this time, the diarrhea had already been present from one to two days. On this account, the difference between the treated and the untreated control pigs was not outstanding. The duration of diarrhea in pigs 1, 15, and 16 was similar to the control pigs, 2, 17, and 18. In pigs 9 and 10, however, the diarrhea ended after the first day of treatment, while control pigs 11 and 12 continued scouring for two more days (fig. 1).

2) Oöcyst discharge: In the pigs (1, 9, 10, 15, 16) which received curative treatment, the oöcyst discharge, suitable for dilution counts, lasted from three to six

days. In the untreated pigs (2, 11, 12, 17, 18), equivalent oöcyst discharge lasted from seven to eleven days. In addition, the range of highest peak of oöcyst production in the treated pigs was from 50,000 to 245,000, whereas that of the untreated pigs was 157,000 to 796,000.

3) Growth: There was little or no gain in weight noted in the pigs of the treated and untreated groups at the end of the second week of infection. This was probably due to the fact that the disease was well under way before treatment was started. One of the older treated pigs (10) made satisfactory body gain (table 1).

DISCUSSION

The average of the daily oöcyst counts in the pigs which received prophylactic and curative treatment, and their respective controls, are presented graphically in figure 2. It may be seen that the oöcyst output for the two untreated control groups (A and C) follow, as it would be expected, a somewhat similar pattern. In these pigs, the oöcyst discharge started about the fifth or sixth day after experimental infection and reached the highest peak on about the eighth day. Thereafter, there was a sharp drop and only a few oöcysts were found after about the seventeenth day. In contrast with the above, in the group of pigs which received sulfaguanidine prophylactically (*B*) there was only a slight oöcyst discharge near the end of the second week of infection. This oöcyst production was largely from pigs 5 and 7, which received the drug for only seven days. This indicates that treatment up to that period was not sufficiently adequate to prevent oöcyst production. However, this method was apparently adequate in preventing the usual symptoms of coccidiosis such as diarrhea and loss of appetite. A point of interest in these 2 pigs (5 and 7) is the absence of oöcyst production, which persisted even up to about one week after the last treatment (*see* fig. 1). It is probable that this was correlated with the prolonged action of the drug in the intestinal tract, which served to inhibit oöcyst production for a few days after the last administration. However, this was not entirely true since a few oöcysts were found in pig 3 beginning with the last day of treatment, and in pig 14 one day after treatment.

The average peak of oöcyst production in the pigs which received curative treatment, as indicated in figure 2 (D), was sharply reduced to a level of about one-third that of the control group (C). In addition, the average period of oöcyst output in the treated group reached a low level on about the tenth day, in contrast with the thirteenth day in the untreated control.

As already noted, in the pigs which received curative treatment, the drug was administered after the diarrhea had started. It should be pointed out that this condition might have been prevented if the drug had been administered earlier. However, curative value of the drug in controlling the diarrhea *per se* was noted in pigs 9 and 10, in which the diarrhea terminated one day after the first administration; in the control pigs 11 and 12, the diarrhea persisted for two more days (*see* fig. 1). In this regard, it is of interest to note that pig 4, which had a prolonged case of diarrhea, was apparently cured of this condition following administration of sulfaguanidine for three days.

The above observations indicate that sulfaguanidine is beneficial in swine coccidiosis produced by *E. debliecki* and *E. scabra*. Although satisfactory results were obtained with this drug with the amount and period given, it is yet to be determined whether or not as good or better results may be obtained if the drug is fed in smaller doses and at different periods.

SUMMARY AND CONCLUSIONS

In the course of six experiments, each of 18 young pigs was experimentally infected with 20 to 30 million oöcysts from mixed cultures of *Eimeria debliecki* and *Eimeria scabra*. Five of the pigs were used to determine the prophylactic value of sulfaguanidine, 5 to determine its curative value and 8 as controls.

The treated pigs received the drug, mixed with feed, at the rate of 1 Gm. per 10 pounds of body weight. Prophylactic treat-

ment started two days before experimental coccidial infection and continued on the day of infection and for seven or ten more days. In the curative treatment, the drug was administered for three days starting with the second day of oöcyst discharge.

In the untreated control pigs, oöcyst output started about the fifth or sixth day after experimental infection, reached a high level on about the eighth day, and dropped to a very low level on about the seventeenth day.

Of the 3 pigs which received the drug prophylactically for a period of ten days following experimental infection, 1 failed to show oöcysts in the feces and 2 others showed only very few oöcysts near the end of the treatment. Two other pigs, which received the drug for seven days following experimental infection, showed a low oöcyst discharge (but higher than that of the above 3 pigs) near the end of the second week of infection.

With pigs which received curative treatment, the oöcyst output was considerably less than that of the control animals, and the period of oöcyst production was also curtailed.

All the pigs which received prophylactic treatment remained normal and made satisfactory body gains. Untreated control pigs developed diarrhea, lost appetite, and made either little or no body gain.

Most of the pigs which received curative treatment made little or no body gain; however, 1 animal made a satisfactory gain. The failure of most of these animals to gain was probably due to the fact that they were treated after the disease was well established.

The results of some of the experiments indicated that diarrhea *per se* was controlled as a result of the sulfaguanidine therapy.

It is concluded that prophylactic use of sulfaguanidine inhibits the life cycle of two species of swine coccidia and prevents symptoms associated with the disease. When given as a cure, the drug brings about a reduction in oöcyst output, thus preventing the spread of the disease, and may also bring about a quicker termination of the diarrheal condition.

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Experiments on the Pathogenicity of Hookworm (Bunostomum Trigonocephalum) Infections in Lambs Fed an Adequate Diet

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BUNOSTOMUM TRIGONOCEPHALUM is the common hookworm parasite of sheep in the United States and in many other parts of the world. Curtice[4] and some other pioneer veterinary parasitologists regarded it as a pathogen causing serious injury to sheep. In a later period, according to Beller[1] and Velu and Balozet,[12] the tendency, especially of German and French investigators and textbook authors, was to hold its pathogenicity in doubt, or to regard it as a harmless parasite, or to ignore it entirely.

About twenty years ago, Beller[1], and Velu and Balozet[12] independently observed and investigated, in large flocks, outbreaks of disease which they reported were due primarily or exclusively to infections with this hookworm. They stated that heavy death losses occurred in the affected flocks.

Their descriptions of the gross pathology observed at necropsy of infected animals were similar in many respects. These authors also agreed regarding infections with the parasite as the cause of anemia, chiefly induced by the sucking of blood by the worms, loss of weight and of wool, progressive weakness, and paralysis followed by death. Beller stated that the degree of anemia observed by him did not suffice to explain the severity of the disease; however, he reported that death was due to hydremic cachexia. He[2] believed that the paralysis observed was due to toxic action of the worms on the central nervous system. Velu and Balozet differentiated between the characteristics of bunostomiasis in adult and in young sheep. In adults, the symptoms were those usually observed in anemia due to parasites. In young animals, the symptoms of anemia were less striking and were overshadowed by nervous manifestations; the course of the disease was more rapid so that at death the animals, though literally bled white, still were in good flesh. These authors believe that the worms elaborate a hemolytic toxin that plays an accessory rôle in the production of the anemia of bunostomiasis.

The conclusions which these investigators reached regarding the pathogenicity of Bunostomum and the characteristics of bunostomiasis have not been adequately tested experimentally. So far as the writers are aware, Beller[2] and Ortlepp[10] are the only investigators who have reported observations on experimentally infected animals. Beller's experiment concerned chiefly the routes of infection. The sheep and goats used by him, as Ortlepp has pointed out, obviously were already infected with Bunostomum when larvae were administered to them. Aside from this restriction on their validity, Beller's observations were very limited in scope. Ortlepp's study also was on the life history of the species, but he briefly described the postmortem findings in one experimental lamb.

Accordingly, our investigation was undertaken to determine the effects of heavy experimental Bunostomum infections on lambs kept under controlled conditions.

Although reports in the literature indicate that sheep may be infected with Bunostomum either by the oral or percutaneous route, they do not reveal which route of infection was the more efficient. Information concerning the number of larvae

From Zoölogical Division, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture.

This work was carried out in 1943 and 1944 at the United States Department of Agriculture Beltsville Research Center, Beltsville, Md.

Some of the principal results of this investigation have been reported in abstract form by the writers.[9]

TABLE 1—Larvae Administered to Test Lambs and Worms Recovered at Necropsy from Four Lambs in Experiment 1

Group No.	Lamb No.	Dates larvae were administered	Method of administration	Hookworm larvae: Administrations	Hookworm larvae: Per administration	Hookworm larvae: Total	Date of slaughter of lambs	Interval from start of experiment to date of slaughter	Worms recovered at necropsy: Strongyloides papillosus	Nematodirus sp.	Cooperia sp.	Trichuris sp.	Ostertagia sp.	Capillaria sp.	Hookworms (Bunostomum trigonocephalum): Males	Females	Total	Relation of hookworms recovered to larvae administered
		1943		No.	No.	No.		Days	No.	No.	No.	No.	No.	No.	No.	No.	No.	%
1	246	6-21	on skin	1	50,000	50,000	10-20-43	121	142	9	0	0	1	0	491	1,016	1,507	3.014
	242	6-21	on skin	1	5,000	5,000	10-20-43	121	238	0	0	4	0	0	246	286	532	10.640
	238	6-21, 28; 7-5, 12, 19, 26; 8-2, 9, 16, 23	on skin	10	500	5,000[1]	[2]	...	...	...	...	...	...	...	...	...	...	...
2	221	6-21	orally	1	50,000	50,000	10-20-43	121	33	5	0	1	0	0	5	1	6	0.0120
	226	6-21	orally	1	5,000	5,000	[2]	...	...	...	...	...	...	...	...	...	...	...
	243	6-21, 28; 7-5, 12, 19, 26; 8-2, 9, 16, 23	orally	10	500	5,000[1]	[2]	...	...	...	...	...	...	...	...	...	...	...
3	215	[3]	...	...	...	...	10-20-43	121	396	0	0	1	0	3	0	0	0	...
	249	[3]	...	...	...	...	[2]	...	...	...	...	...	...	...	...	...	...	...

[1]A total of approximately 190 Strongyloides larvae also administered.
[2]Not slaughtered in experiment 1; used again for experiment 2.
[3]Control lamb; no larvae administered.

TABLE 2—Results of Examinations of Feces of Lambs in Experiment 1 for Helminth Eggs and Coccidial Oöcysts

Group No.	Lamb No.	Bunostomum eggs per gram of feces found on examination carried out on indicated day after start of experiment[1]: 60	67	74	81	88	95	102	109	116	Average number of Bunostomum eggs per gram[2]	Average number of Strongyloides eggs per gram[3]	Index of average density of oöcysts[4]
		No.	No.	No.	No.	No.	No.	No.	No.	No.			
1	246	222	1,204	3,200	4,000	3,800	6,240	2,800	3,200	2,130	2,977.33	256.42	1.50
	242	186	450	1,400	1,800	2,240	3,000	1,080	1,800	930	1,436.22	292.42	1.29
	238	10	40	154	400	176	667	400	680	400	325.22	201.58	1.24
Av.	...	...	...	...	...	...	...	...	...	...	1,579.59	250.13	1.34
2	221	0	0	0	1	6	16	8	6	8	7.50	114.74	1.53
	226	0	0	0	0	0	0	0	0	0	0.00	1,408.21	1.58
	243	0	0	0	6	12	6	12	6	8	8.33	146.94	1.82
Av.	...	...	...	...	...	...	...	...	...	...	5.28	556.30	1.65
3	215	0	0	0	0	0	0	0	0	0	0.00	494.95	1.26
	249	0	0	0	0	0	0	0	0	0	0.00	264.31	1.53
Av.	...	...	...	...	...	...	...	...	...	...	0.00	379.63	1.39

[1]Examinations carried out 10 and 3 days before larvae were administered to the test lambs and 4, 11, 18, 25, 32, 39, 46 and 53 days thereafter, were negative for Bunostomum eggs for all lambs.
[2]For infected lambs, average of positive counts.
[3]Average of 19 counts carried out simultaneously with Bunostomum egg counts.
[4]Average of 19 density estimates carried out simultaneously with Bunostomum egg counts; oöcyst density per coverslip was recorded as one-half plus, plus, one and one-half plus, etc., but for convenience is expressed numerically.

required to produce a heavy experimental infection also was lacking. Likewise, it was not apparent whether a given number of larvae would yield more worms if administered at one time or in serial apportionments at brief intervals over a considerable period of time.

Consequently, in seeking to obtain infections suitable for the purposes of the investigation, it was considered desirable to test both routes of infection, to vary the number of larvae given to test animals and to compare the results of massive single administrations and smaller serial administrations of larvae.

the test lambs were isolated routinely from cultures of feces from ram 166. This ram had been raised free of helminths except Strongyloides. It was infected with hookworms experimentally by placing on its skin infective larvae raised from eggs removed from female *B. trigonocephalum*. A short time before the investigation began, the ram was superinfected with larvae isolated from cultures of its own feces. Exceptionally, larvae were obtained from the feces of a second ram harboring a hookworm infection derived in turn from the feces of ram 166.

The various cultures from which larvae were isolated for the experiments had been prepared from five to twenty-one, usually seven to fourteen days previous to the time they were placed in the Baermann apparatus. Usually, the lar-

TABLE 3—Summary of Values of Indexes of Anemia for Lambs in Experiment I

Group No.	Lamb No.	Observations No.	Hematocrit (packed red cell volume) Max. %	Min. %	Av. %	Hemoglobin (per 100 cc.) Max. Gm.	Min. Gm.	Av. Gm.	Red cell Count (per cmm.) Max. Millions	Min. Millions	Av. Millions
1.....	246	19	34.0	18.5	25.16	12.0	6.0	8.58	12.00	5.92	8.87
	242	19	37.5	27.0	30.46	12.0	8.5	10.03	12.57	8.39	10.03
	238	19	36.0	29.0	32.33	12.0	10.0	11.08	13.40	10.40	11.75
Av.	...	..	....	....	29.32	....	....	9.90	.....	.....	10.22
2.....	221	19	36.0	31.0	33.74	12.0	10.0	11.16	11.75	10.18	10.98
	226	18	35.5	31.5	33.67	12.5	10.0	11.28	13.87	9.69	12.66
	243	18	40.5	32.0	37.22	14.0	11.5	12.42	13.37	10.18	12.48
Av.	...	..	....	....	34.88	....	....	11.62	.....	.....	12.04
3.....	215	19	37.5	29.0	32.33	12.0	10.0	10.97	13.95	10.20	11.79
	249	18	38.0	31.0	36.03	13.0	11.0	12.06	14.12	11.17	12.40
Av.	...	..	....	....	34.18	....	....	11.52	.....	.....	12.09

MATERIALS AND METHODS

Animals, Housing, and Diet.—Eight weaned male lambs of mixed breeding about 4 to 4½ months old, born and raised in frequently cleaned indoor concrete pens, were selected for this work. They were separated into groups, as indicated elsewhere, and the various groups were kept in separate concrete pens in a barn. Attached to each of the pens was a small, fenced, concrete-floored, outdoor runway to which the occupants were given access in good weather. The pens and runways were cleaned out thoroughly at forty-eight- to seventy-two-hour intervals, except in winter when the interval between cleanings was seventy-two to ninety-six hours.

The lambs were fed at the rate of ½ lb. of mixed grain (usually, 17.5 per cent cracked corn, 75 per cent whole oats, and 7.5 per cent bran) and about 3 lb. of U. S. No. 2 alfalfa hay per head per day; rock salt and water were available to them at all times.

Infective Larvae and Administration.—The infective Bunostomum larvae administered to vae were used on the same day the cultures were placed in the isolation apparatus; exceptionally, they were kept in water as long as three days before use. In estimating the number of larvae in the various suspensions, the usual dilution method of counting was followed; only active larvae were counted. All larvae administered on a given day were from the same suspension.

For oral administration, larvae suspended in a few cc. of water were forcibly expelled onto the base of the tongue by means of a pipette held well back in the lamb's mouth. After swallowing had been induced, a small volume of rinse water was similarly given. Larvae were applied to the skin, after suspension in the least possible volume of water, by means of a pipette; after the suspension was spread over a wool-free area, usually under a foreleg, the lamb was kept in restraint until the water had evaporated. In the case of repeated administrations, the larvae were applied alternately beneath the right and left foreleg. If, as often was the case, inflammation resulting

from the previous application still was present in part of the skin area, the larvae were, so far as possible, applied to the normal or least inflamed remaining portions.

Many of the cultures used as a source of hookworm larvae also yielded large numbers of Strongyloides larvae, as both of the source animals harbored *Strongylus papillosus*. Efforts to kill the Strongyloides larvae selectively by prolonged storage of the mixture of larvae in water, or by brief desiccation, indicated that exposures fatal to them seriously damaged the hookworm larvae. Under these circumstances administration of large numbers of Strongyloides larvae to certain of the test lambs was unavoidable.

Clinical and Laboratory Examinations.—For fifteen weeks before experiment 1 began, and throughout the investigation, the lambs were weighed at regular weekly intervals. In the week before experiment 1 began, and weekly during this experiment, a sample of feces and about 5 cc. of blood from the jugular vein were collected from each lamb, and rectal temperatures were taken. Samples were taken and temperature readings were made weekly during part of experiment 2 and biweekly during the remainder of this experiment, as indicated elsewhere. The lambs frequently were observed for signs of inappetence, diarrhea, or acute illness; during part of experiment 1 and all of experiment 2, the color of the skin and con-

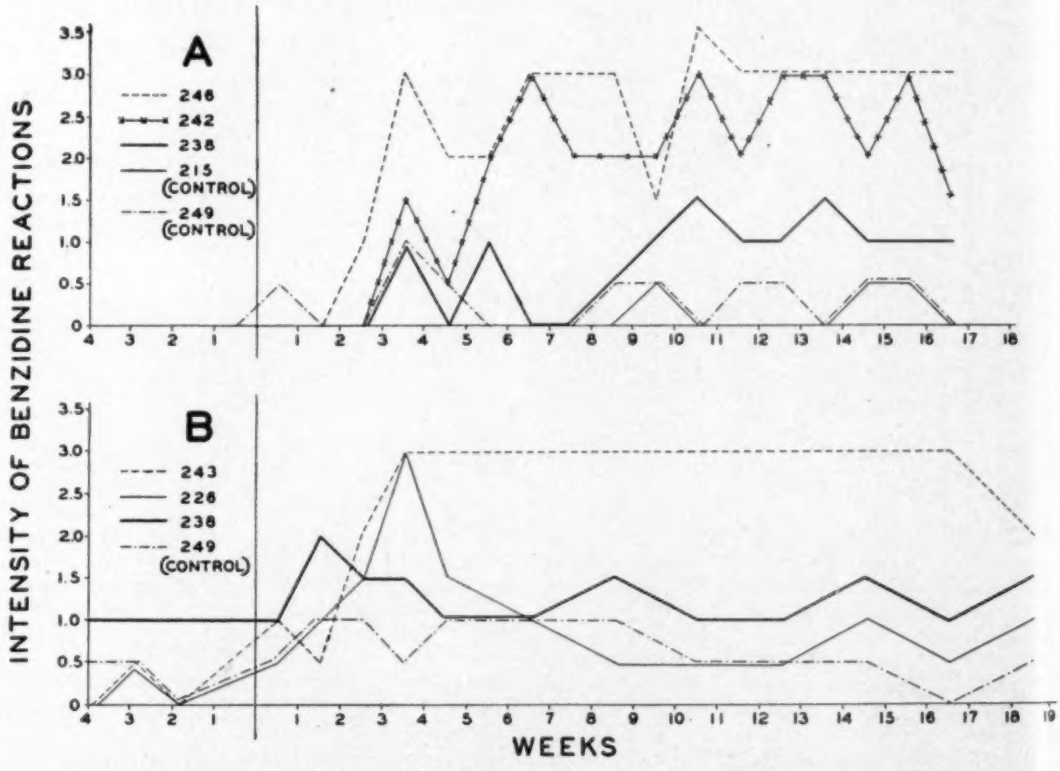

Fig. 1, A-B—Intensity of color reactions in benzidine tests of feces of lambs; presence of occult blood in the feces of infected lambs is indicated only by reactions more intense than shown for the control lambs. (In each graph the long vertical line denotes the 0-point on the time-scale or initiation of the experiment by administration of larvae to the test lambs).

A—Reactions for lambs of group 1 (those for lambs of group 2 are not shown; see text) and for control lambs in experiment 1 (the first tests were made 3 days before infection; incompletion or interruption of a curve indicates that more than 2 successive negative reactions were recorded).

B—Reactions for lambs in experiment 2.

(Significance of numerical expressions of reaction intensity: 0 = no reaction or slight trace of color; 0.5 = greenish or very light blue color developing slowly and persisting a few seconds; 1.0 = light blue or greenish-blue developing in about ten seconds and persisting about ½ minute; 1.5 = light blue to blue developing in about five seconds and persisting about one minute; 2.0 = blue developing immediately and persisting about two minutes; 3.0 = blue developing immediately and changing to purplish-blue persisting about five minutes; 3.5 = blue developing immediately and changing to deep blue-black persisting more than ten minutes.)

TABLE 4—Summary of Total Leucocyte Counts on Blood of All Lambs in Experiment I and Proportions of Five Types of Leucocytes Counted Differentially in Blood Smears from Two Infected Lambs and One Control Lamb

Group No.	Lamb No.	Total leucocytes per cmm. of blood: In count in week before infection of test lambs	Total leucocytes per cmm. of blood: Average and extremes[1] of 17-18 weekly counts during observation period		Average and extremes[1] of percentages of indicated types of leucocytes and of smudges in 16 to 19 differential counts usually at weekly intervals: Lymphocytes (%)	Neutrophiles (%)	Eosinophiles (%)	Monocytes (%)	Basophiles (%)	Smudges (%)
		Thousands	Thousands							
1	246	9.06	7.26	(5.10-11.70)	65.77 (39.24-78.60)	26.86 (16.20-56.57)	1.94 (0.40-5.00)	3.10 (0.60-5.45)	0.87 (0-2.28)	1.48 (0-4.60)
	242	11.40	8.74	(6.05-12.40)	72.37 (58.20-83.20)	23.11 (12.00-35.20)	0.65 (0-2.20)	1.86 (0.60-4.00)	0.69 (0-1.77)	1.20 (0-3.40)
	238[2]	8.54	6.64	(4.10-9.25)	.....	.....	....	....	....	....
Av.	...	9.67	7.50							
2	226[2]	12.26	9.11	(6.65-12.26)	.....	.....	....	....	....	....
	221[2]	10.64	9.77	(5.65-18.00)	.....	.....	....	....	....	....
	243[2]	8.34	9.20	(5.95-13.10)	.....	.....	....	....	....	....
Av.	...	10.41	9.36							
3	215	14.26	10.03	(6.95-14.26)	78.33 (57.40-89.40)	18.49 (9.20-38.60)	0.30 (0-1.00)	1.10 (0-2.60)	0.30 (0-1.00)	1.47 (0.40-3.60)
	249[2]	8.86	8.25	(5.70-14.50)	.....	.....	....	....	....	....
Av.	...	11.56	9.14							

[1]Indicated by figures in parentheses.
[2]Differential counts not made.

junctiva and the comparative general appearance and condition were observed at the regular intervals mentioned.

Within an hour of collection, the fecal samples were tested for the presence of occult blood. For a few tests, hydrogen peroxide and a 1 per cent solution of benzidine in glacial acetic acid were the reagents. Routinely, however, the test was carried out as follows: A small bit of feces carefully removed from the interior of a pellet was placed in the well of a hanging-drop slide and thoroughly mixed with enough 50 per cent acetic acid to fill the well; upon the surface of this mixture was dropped a small amount of a powder prepared in a mortar by triturating benzidine and barium dioxide in the proportion of 0.025 Gm. of the former to 0.2 Gm. of the latter. In every test, comparable amounts of feces and of the powdered reagent were employed. Since the benzidine reaction is the basis of a colorimetric quantitative method for the determination of hemoglobin, it was considered permissible to grade the color reactions comparatively and numerically on the basis of speed of development, intensity as shown by depth of color developing in the range from light greenish-blue to blue-black, and persistence. The values so obtained were regarded as affording a rough index of comparative blood loss. Tests made on feces to which had been added known quantities of diluted blood of known hemoglobin content indicated that the test, as carried out, was sensitive, though having a somewhat variable threshold, and was affected in the characteristics mentioned in accordance with the amount of hemoglobin added. This test does not distinguish between hemoglobin and chlorophyll. Very likely because of the presence of the latter substance in the alfalfa hay[1] fed to the lambs, the feces of the hookworm-free controls frequently gave a slight, and, more rarely, a definitely positive, reaction. Therefore, a color reaction more rapid, more intense, and more persistent than any obtained with the feces of a control lamb was the minimum indication of the presence of blood in the feces.

All fecal samples were examined by a quantitative flotation method. The comparative density of coccidial oöcysts per coverslip was noted. If the helminth eggs adhering to the coverslip were not too numerous to be counted with reasonable accuracy, the number of each kind present per gram of feces also was ascertained; otherwise, an additional or alternative count of the eggs was made by a standard dilution method.

The oxalated blood specimens were used for hemoglobin (Haden-Hausser clinical hemoglobinometer) and hematocrit (Wintrobe) determinations, for total red cell and white cell counts, and for films (Wright's stain) for differential leucocyte counts (500 cells). All determinations and counts were made by the same observer.

Examinations at Necropsy.—At the time of slaughter, the contents of the abomasum and small intestine of each lamb were preserved after screening and sedimentation; later this material was searched carefully for worms and those found were identified and counted. An immediate examination of the cecum and large intestine for whipworms, nodular worms, and hookworms was similarly carried out; the large gut was searched for nodular worm lesions. Observations on the location and attachment of hookworms were carried out. All organs were examined for evidence of gross lesions. The skinned, eviscerated carcasses, pelts, and certain organs, as indicated elsewhere, were weighed.

EXPERIMENT 1

Infection of Lambs.—The lambs were separated into two test groups of 3 animals each, and a control group of 2. Individuals were allocated so that the 3 groups were, so far as possible, comparable in average age and weight. Infective larvae were placed on the skin of the lambs of 1 test group (group 1), but were given orally to the lambs of the second test group (group 2), as indicated in table 1.

Occult Blood in the Feces.—Except for promptly appearing reactions of the skin in areas to which larvae were applied, the appearance of blood in the feces of certain of the test lambs was the first evidence of infection. An intense benzidine reaction was obtained in the feces of lamb 246 (group 1) on July 16, twenty-five days after infection; one week earlier the reaction was more intense than for either of the control lambs that day, but this fact is only suggestive of the possibility that bleeding occurred within eighteen days of infection. The feces of lamb 242 (group 1) also gave, twenty-five days after infection, a reaction of an intensity indicative of the presence of hemoglobin. Throughout the remainder of the experiment, there was evidence of marked blood loss in the feces of lamb 246 (fig. 1A) and significant reactions also continued to be obtained with the feces of lamb 242. On two examinations (fig. 1A), more intense reactions were obtained in the

[1] In tests carried out by the same method in a later investigation, the feces of hookworm-free lambs fed timothy hay uniformly gave no reaction.

feces of lamb 238 than ever were observed for either control lamb, but loss of blood in the feces of this lamb apparently was not marked.

No definite evidence was obtained of blood loss in the feces of any of the lambs of group 2.

Egg-count Data.—The next evidence of hookworm infections in the test lambs was afforded by the results of the weekly fecal examinations.

At the beginning of the experiment, all of the lambs harbored Strongyloides and coccidial infections only. So far as was revealed by the preliminary examinations and by those carried out during the experiment, the average intensity of coccidial infections differed among the various lambs only in minor degree (table 2). Clinical coccidiosis did not occur in any of the lambs.

So far as was indicated by egg counts (table 2) the level of Strongyloides infection was lower in all test lambs, except lamb 226, than in control lamb 215. During the experiment, lamb 226 showed the highest average Strongyloides egg count; however, as indicated elsewhere, its infection had no demonstrable effect on its red blood cell values or general health. On a few examinations, eggs of Trichuris or of Cooperia were found in the feces of lambs 243 and 242; maximum counts of two such eggs per gram indicated low grade infections with these helminths.

Hookworm eggs first were found in the feces of all 3 lambs of group 1, sixty days (table 2) after infection; the prepatent period in these infections, therefore, was between fifty-three and sixty days. The hookworm egg-count levels and averages indicated that the degree of infection throughout the remainder of the experiment was greatest in lamb 246, which received a single cutaneous application of 50,000 larvae. Lower egg counts were obtained for lamb 238 than for lamb 242, although each received 5,000 larvae. However, this fact does not necessarily signify that fewer hookworms developed in lamb 238 than in lamb 242, for the former received ten equal weekly applications of larvae and the latter only a single application given on the day lamb 238 received its first apportionment of larvae.

Hookworm eggs first were found in the feces of lambs 221 and 243 (group 2) eighty-one days after the start of the experiment (table 2), twenty-one days later than in the case of the percutaneously infected lambs. Throughout the experiment, the egg count level was very low for these lambs, indicating low levels of infection. No hookworm eggs were found in the feces of lamb 226 of group 2.

Since the sensitivity of the technique of fecal examination was no greater than two eggs per gram, it cannot be concluded with certainty that development to maturity required longer following oral than following percutaneous entry of larvae. For the same reason, the negative finding in lamb 226 is to be regarded as relative rather than absolute.

Obviously, however, in this experiment infection by the percutaneous route was far more efficient than by the oral route.

Indexes of Anemia.—The values of the weekly hematocrit, hemoglobin, and red cell count determinations made on the blood of the control lambs (215 and 249) fluctuated considerably during the experiment (fig. 2, A, B, C,), but showed no definite directional trend. For the purposes of this experiment, the lower limit of the normal range of each index is regarded as set by the minimum value of each recorded for the lambs of the control group (table 3).

The lowest values (table 3) recorded for the 3 lambs of group 2 and for lamb 238 (group 1) were higher, or only very slightly lower, than the respective minimums of normality established by the data for the controls; furthermore, the weekly values for these 4 test lambs fluctuated with as little evidence of trend as did those for the controls. Thus, it is evident that lambs 226, 221, 243 and 238 showed no anemia. Curves representing the weekly values for these 4 lambs are not included in figure 2 because it was apparent that, if shown, they would have merely complicated the graphs needlessly.

TABLE 5—Weights of Lambs and Rates of Change in Weights Before and During Experiments 1 and 2

Group No. in experiment 1	Lamb No.	Weight of lambs in experiment 1				Group No. in experiment 2	Weight of lambs in experiment 2		Net gain in weight in experiment 2 (19 weeks)	Rate of gain in lb. per week in—			Increase (+) or decrease (—) in rate of gain in lb. per week	
		At first weighing (3-6-43)	Two days before start of experiment (6-19-43)	Four days before end of experiment	Net gain in weight in experiment 1 (17 weeks)		One day after start of experiment (10-30-43)	Six days before end of experiment (3-11-44)		Fifteen weeks before experiment 1	Experiment 1	Experiment 2	In experiment 1[1]	In experiment 2[2]
		lb.	lb.	lb.	lb.		lb.	lb.	lb.	lb.	lb.	lb.	lb.	lb.
1	246	23.75	58.00	76.75	18.75	..	....	.....	....	2.30	1.10	....	—1.20	.....
	242	28.00	68.50	96.00	27.50	..	....	.....	....	2.70	1.60	....	—1.10	.....
	238*	22.00	53.00	73.00	20.00	4	75.0	100.0	25.0	2.00	1.20	1.32	—0.80	+0.12
	Averages	24.91	59.80	81.90	22.08	..	....	.....	....	2.33	1.30	....	—1.03	.....
2	226*	31.75	70.00	96.25	26.25	4	97.5	125.0	27.5	2.60	1.50	1.45	—1.10	—0.05
	221	23.50	52.50	82.00	29.50	..	....	.....	....	1.90	1.80	....	—0.10	.....
	243*	22.00	48.00	70.50	22.50	4	77.0	82.0	5.0	1.70	1.30	0.26	—0.40	—1.04
	Averages[3]	25.75	56.80	82.90	26.08		83.1	102.3	19.2	2.06	1.50	1.03	—0.53	—0.32
3	215	25.00	62.00	94.50	32.50	..	....	.....	....	2.50	1.90	....	—0.60	.....
	249*	23.00	54.50	88.50	34.00	5	92.0	131.0	39.0	2.10	2.00	2.05	—0.10	+0.05
	Averages[4]	24.00	58.25	91.50	33.20	..	92.0	131.0	39.0	2.30	1.95	2.05	—0.35	+0.06

*Lamb carried over into experiment 2. [1]Based on weekly gain in fifteen weeks before experiment 1. [2]Based on weekly gain in experiment 1.
[3]Groups 2 and 4.
[4]Groups 3 and 5.

The blood of each of the remaining 2 lambs (246 and 242) of group 1 showed values lower than the minimums recorded for the controls or for the other 4 test lambs (table 3).

About six weeks after infection, the red cell values of lamb 242 reached levels definitely below the normal range (fig. 2, A, B, C); with a single exception, none recorded thereafter was within the range of normality as set by the data for the control lambs or within the range of values observed for the lambs of group 2 and lamb 238. These facts are considered indicative of a slight degree of anemia in lamb 242.

Within about three to five weeks after infection, the values for lamb 246 declined below the minimums of normality as shown by the data for the control lambs (fig. 2, A, B, C), and thereafter until the eleventh to the fifteenth week of the experiment they showed a more or less straight-line decline to levels approximately one-half the respective average values for the control lambs (table 3), or one-half of the pre-experimental averages for all lambs. These facts clearly indicate the development of a moderately severe anemia in lamb 246.

An upward trend occurred in all values recorded for lamb 246 in the last two to three weeks of the experiment and this indicated that recovery toward normal red cell values was in progress in this lamb when the experiment ended.

Calculation of the mean corpuscular volume and hemoglobin and mean corpuscular hemoglobin concentration (Wintrobe and Buell[13]) from each of the 19 available sets of observations on the blood of lamb 246 indicated that the anemia of this lamb was essentially normocytic, but possibly hypochromic. From about the sixth to the twelfth week after infection, both corpuscular volume and corpuscular hemoglobin were greater than before the lamb was infected; thereafter the value of both constants declined. The corpuscular hemoglobin and hemoglobin concentration reached levels lower than were recorded early in the experiment, but this was not true of

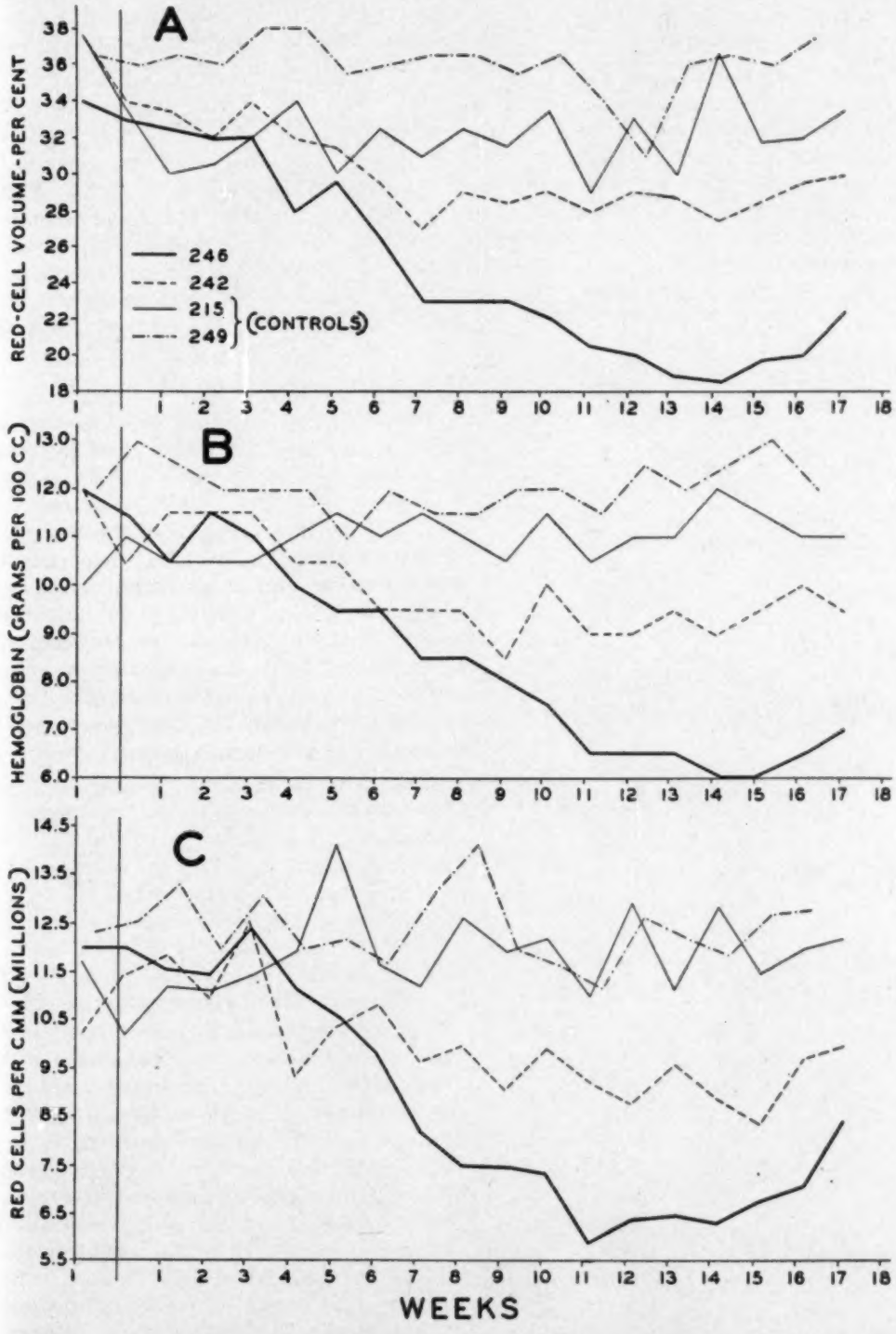

Fig. 2, A-C—Weekly hematocrit and hemoglobin determinations and red-cell counts on blood of 4 lambs in experiment I. (In each graph the long vertical line denotes the 0-point on the time-scale or initiation of the experiment by administration of larvae to the test lambs.)

corpuscular volume. When the anemia was most intense, each of the three constants had approximately the value of the corresponding constant for lamb 215, 1 of the controls; this was true whether the calculations for lamb 215 were based on the average red cell count, hemoglobin, and hematocrit, or on the readings for the day when the anemia of lamb 246 was greatest.

Leucocytes.—Weekly counts showed that the total number of leucocytes in the blood of the lambs was not affected by Bunostomum infection. Comparison of the initial count and the average of all counts for each of the lambs, as shown in table 4, indicated that the greatest difference between these figures occurred in the case of control lamb 215. A general tendency for the number of leucocytes in the blood of each lamb to decline with progress of the experimental period was suggested by the complete series of counts for the various lambs; this trend is not evident from the summary figures given in the table.

Differential white cell counts were not made for any lamb of group 2, since other data of the experiment suggested that this would be unprofitable. Such counts were made, however, for 3 of the lambs, a control (215) and the 2 most heavily infected lambs (246, 242) of group 1. The results (table 4) indicated occurrence of an eosinophilia in the infected lambs; its degree was greater in the more heavily infected one (246). The data also suggest that infection caused a slight monocytosis and basophilia. As indicated graphically in figure 3, A, the complete series of eosinophile percentages for lamb 246 failed to indicate any definite peak in the eosinophile response of this, the most heavily infected, lamb.

Worms Recovered at Necropsy.—This experiment was concluded by slaughter of 4 of the 8 lambs on Oct. 20, 1943. The remaining 4 lambs were kept for a second experiment for the reasons and purposes stated later.

Lambs 246 and 242 were killed to allow determination of the numbers of hookworms responsible for the degree of anemia observed in each of them. Lamb 221 was killed so that the number of hookworms developing in it from 50,000 orally administered larvae could be compared with the number developing in lamb 246 from 50,000 percutaneously administered larvae. Lamb 215, a control, was killed for comparative purposes.

Lamb 246 harbored at necropsy approximately 1,500 hookworms, lamb 242 approximately 500, and lamb 221 only six (table 1). These worm counts were positively correlated with the hookworm egg counts (table 2) recorded for the respective lambs.

Thus, about four months after infection, about 250 times more hookworms were found in lamb 246 than in lamb 221. This showed, as the egg counts had indicated earlier, that Bunostomum larvae infected far more efficiently by the percutaneous than by the oral route.

Lamb 246 harbored only about three times as many hookworms as lamb 242, in spite of the fact that it received, in a similar manner, ten times as many larvae. This indicated a diminishing percentage return of adult worms with increase in number of larvae applied (table 1) to the skin.

Other nematodes found at necropsy included Strongyloides, Nematodirus, Trichuris, Ostertagia, and Capillaria. Only Strongyloides was found in appreciable numbers in any lamb. The control lamb, which was not anemic, harbored the largest number of Strongyloides. Hence, none of these extraneous infections is regarded as having affected the main results of the experiment.

Relation between Egg Counts, Worms Recovered, and Anemia.—Lambs 246 and 242 (group 1) each had received a single application of larvae. Lamb 246, which received the larger number of larvae, became more anemic than lamb 242, passed more hookworm eggs in its feces, and harbored more hookworms at necropsy.

Lamb 238 (group 1), which was not killed in this experiment, did not become definitely anemic; the egg counts recorded for it were higher than those for lamb 221, but not as high as those for lamb 242.

Lamb 221 (group 2) did not become anemic; very few hookworm eggs were found

in its feces and very few hookworms were found in it at necropsy.

The 2 remaining lambs of group 2, which were not killed at the end of this experiment, did not become anemic; either very few, or no, hookworm eggs were found in their feces.

Influence of Infection on Weight Gains. —The maximum individual and group average weight gains were made by the control lambs. The weekly gain of each of the 8 lambs was less during the experiment than during the fifteen-week pre-experimental period. However, the lambs of group 1 showed a greater decline in gain per week than did either control lamb; also, on the average the lambs of group 2 showed a greater decline than the control group. These facts may seem to warrant the general statement that Bunostomum infection adversely affected gain in weight. So sweeping a conclusion actually is not justified because the lambs were of mixed breeding, their weights at the start of the experiment covered a wide range, and the weight performance of certain lambs of groups 1 and 2 was inconsistent with that of other lambs of these groups, taking into account the comparative degree of infection among them.

Nevertheless, careful consideration of the individual characteristics of the lambs, the trends in their weekly weights (fig. 4, A), and the data in table 5 has led the writers to the conclusion that the poor gain of lamb 246 during the experiment was largely due to this lamb's heavy infection. In the fifteen-week pre-experimental period, lamb 246 outgained 4 of the remaining 7 lambs, including control lamb 249. It was 3.5 lb. heavier than lamb 249 when the experiment began. About six weeks later (fig. 4, A) the control lamb had more than made up this weight differential. In the next nine weeks, lamb 246 gained only 5.5 lb., while lamb 249 gained 14.25 lb. For one three-week interval in this period, the weight of lamb 246 remained almost stationary, but throughout the nine weeks lamb 249 gained steadily. It was precisely while this change in the weight relationship of these 2 lambs was taking place that lamb 246 became definitely anemic and showed a progressive and obvious regression in general appearance and physical condition such as was noted in no other lamb.

Moreover, lamb 246 showed the lightest pelt, carcass, and omentum, the lowest dressing percentage, and the lowest carcass grade of the four lambs slaughtered at the end of the experiment (table 6).

EXPERIMENT 2

Since extreme anemia had not resulted from any of the infections produced in experiment 1, the principal object of the second experiment was to induce infections heavier than any obtained previously. In view of the results of the first experiment, it was decided to infect by skin exclusively and to use large numbers of larvae for infections.

Lambs 226, 243, and 238, which were test lambs in experiment 1, were used again as test lambs (group 4). Lamb 249 was again used as a control (group 5).

Lamb 226 had failed to become infected orally and lamb 243 had become only lightly infected orally in experiment 1, but it was thought both readily could be infected by skin. Since lamb 238 had been repeatedly exposed to skin infection and still showed considerable numbers of hookworm eggs in its feces, the writers thought that administration of more larvae to it would incidentally show if it had acquired resistance to further infection.

Infection of Lambs. — Lamb 226 was given two massive applications of larvae, the second four days after the first (table 7); the total number of larvae it received was about 580,000. Lamb 238 received a roughly similar total number, about 560,000 larvae; however, it was necessary to administer them serially in 11 applications over a period of about one month. Each time lamb 238 was given larvae, lamb 243 received one-fifth as many; thus, it was given in all about 112,000 larvae (table 7).

RESULTS

The chief result was that lamb 243 became heavily infected (tables 7 and 8) and

anemic (fig. 5, A, B, C). It was gradually recovering from its anemia when all 4 lambs were slaughtered about nineteen weeks after the experiment began.

Although lamb 243 passed more hookworm eggs in its feces and yielded at necropsy about 500 more hookworms than lamb 246 had in experiment 1, the lowest levels recorded for the three indexes of anemia were nearly identical for the 2 lambs in question. Calculations showed that the anemia of lamb 243 also was similar in type to that of lamb 246. In experiment 2, lamb 243 was the poorest weight-gainer, as lamb 246 was in experiment 1.

Since the main results of the two experiments are generally comparable, it is not necessary to describe the results of experiment 2 in detail. Some features of special interest or of difference will, however, be mentioned under the headings that follow:

Occult Blood Tests.—As in experiment 1, hemoglobin-benzidine reactions of significant intensity were first obtained for 2 of the lambs, 226 and 243, about twenty-five days after the experiment began (fig. 1, B). Perfectly correlated with other data of the experiment were the following circumstances: (1) for lamb 243, the reactions remained at a high level of intensity almost to the end of the experiment; (2) but in the case of lamb 226, they quickly declined in intensity so that at the seventh week of the experiment, and thereafter, they were similar to those recorded for the control lamb; (3) no relation was apparent between the administration of larvae to lamb 238 and the subsequent intensity of the reactions of its feces.

Hookworm Egg Counts. — The further administration of larvae to lamb 238 resulted in no increment in the numbers of eggs in its feces. The highest count in its infection history (tables 2, 8) was obtained before any worms resulting from these larvae could have reached maturity. However, this lack of effect on egg count is not construed as proof of acquired resistance because of the behavior of lamb 226, which also showed comparatively few eggs in its feces despite the huge number of larvae given it. That age was not a factor in the insusceptibility of these 2 lambs, was indicated by the high egg counts obtained in the feces of lamb 243. This lamb received the smallest individual applications and total number of larvae. Hence, it is believed, the results in lambs 226 and 238 may for practical purposes be considered to indicate that by their very numbers the larvae administered to these lambs provoked a response which defeated the purpose of inducing heavy infections. At any rate, the objective fact is that extremely large numbers of larvae, whether given as virtually a single massive application or in a series of smaller ones, failed to result in heavy infections in 2 lambs (table 7), 1 of which harbored a moderate preëxisting infection.

The peak of egg production in the infection of lamb 243 occurred, so far as the data indicate, only six days later than in the case of the 2 group 1 lambs receiving a single application of larvae in experiment 1. This may signify that, even in this lamb, only the first few applications of larvae led to the establishment of appreciable numbers of adults.

Hematologic Data. — In the first five weeks of the experiment, all three indexes of anemia for lamb 226 showed a more or less straight line decline (fig. 5, A, B, C) to values indicative of a slight comparative degree of anemia. This decline was nearly as marked as that for lamb 243 in the same interval; thereafter, however, it was arrested and values in the normal range were gradually attained. The red cell values for lamb 238, though usually lower than those for the control, were not, following reinfection, demonstrably affected, since they bore the same comparative relationship to those for lamb 249 as formerly. The degree of anemia produced in lamb 243 has been noted.

In this experiment, as in experiment 1, infection had no apparent effect on the total number of leucocytes in the blood (table 9). Compared to the control, all infected lambs showed a degree of eosinophilia and monocytosis, as was the case for lambs 246 and 242 in experiment 1. The average eosinophile percentages were directly related in magnitude to the hookworm egg

counts and the size of the hookworm infections in the respective lambs, but, in contrast to the findings in experiment 1, this was not true of the monocyte and basophile percentages. In fact, lamb 243 and the control showed about the same average basophile percentage. Both lamb 243 and lamb 238 showed higher eosinophile percentages than were found for lamb 246 in experiment 1. In the case of lamb 238, the peak percentage occurred early in the experiment (fig. 3, B); this suggests prompt response of the host to the newly invading larvae. The possible relationship of this

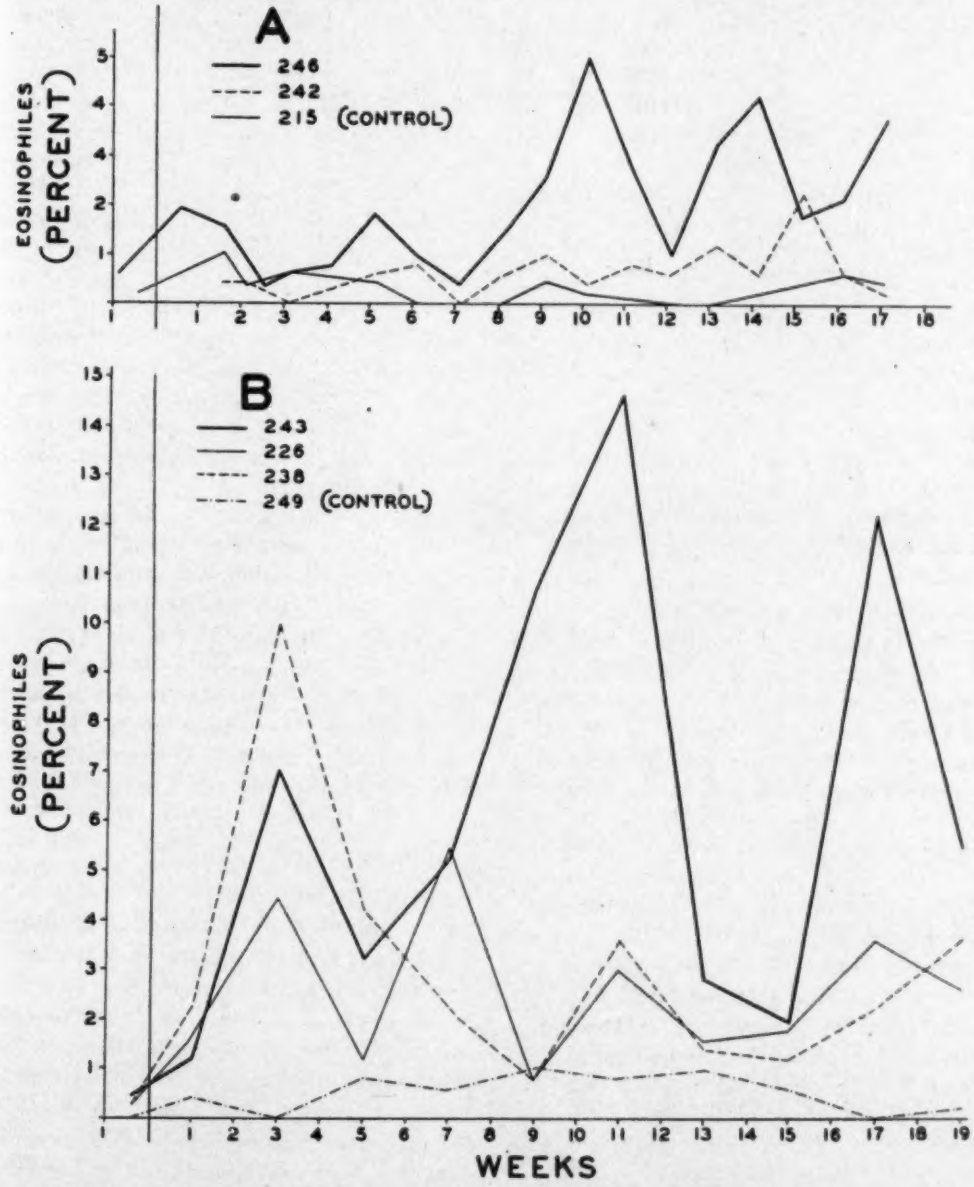

Fig. 3, A-B—Percentages of eosinophiles observed in the blood of certain infected and control lambs (In each graph the long vertical line denotes the 0-point on the time-scale or initiation of the experiment by administration of larvae to the test lambs). A—Percentages observed for two infected lambs and one control lamb in experiment 1. B—Percentages observed for lambs in experiment 2.

fact to this lamb's preëxisting infection and its failure to become reinfected to a demonstrable extent is interesting, but is not clear.

Weight Gains.—The control lamb and test lambs were not of matching weights at the start of the experiment. However, certain comparisons in the gains of individual animals appear to be valid and to reveal more striking evidence of an adverse effect of Bunostomum infection on gain than was obtained in experiment 1.

Lambs 238 and 243 (table 5; fig. 4, B) were of comparable weight at the start of the experiment. Neither had been previously a good gainer, but there was not much difference in their earlier performance, particularly since the midpoint of experiment 1. As already noted, lamb 238 was virtually unaffected parasitologically by the larvae given it in experiment 2 and showed no anemia; its rate of gain was also practically unaffected (table 5). Although during the first eight weeks of experiment 2 (fig. 4, B) heavily infected lamb 243 outgained lamb 238, thereafter it showed a cessation of growth similar to that shown by lamb 246 in experiment 1. At the end of the experiment, it weighed less than at the beginning of the ninth week and was 18 lb. lighter than lamb 238.

At the beginning of the experiment, lamb 226 was 5½ lb. heavier than the control lamb (249); in all earlier periods covered by the data it had been considerably the heavier. However, from the beginning of the third to the end of the fifth week of this experiment lamb 226 failed to gain (fig. 4, B). In this interval, the weight of lamb 249 increased steadily a total of 14 lb. It was precisely in this period that lamb 226 showed marked evidence of blood loss in its feces and declining red cell values. Except in a later period when the weight of lamb 249 declined temporarily because of inappetence caused by an acute digestive disturbance, the weight of lamb 226 did not regain its former relationship to that of the control. Despite its illness, lamb 249 weighed 6 lb. more than lamb 226 at the last weighing; just before lamb 249 became sick, its weight was the greater by 13 lb.

TABLE 6—Slaughter Characteristics of the Experimental Lambs

Group No.	Lamb No.	Live weight at slaughter after fasting for 24 to 48 hours	Weight of—			Dressing percentage	Carcass grade[1]
			Pelt	Dressed carcass	Omentum		
		lb.	lb.	lb.	Gm.	(%)	
EXPERIMENT 1							
1	246	69.50	6.75	28.36	162	40.8	Top commercial
1	242	88.25	9.50	41.50	325	47.0	Top good
2	221	75.25	9.75	31.94	353	42.2	Straight good
3	215	85.75	8.00	38.12	387	44.4	Top good
EXPERIMENT 2							
4	226	123.25	16.50	53.00	545	43.8	[2]
4	238	98.50	15.50	44.50	770	45.2	[2]
4	243	79.75	13.00	33.00	430	41.4	[2]
5	249	129.25	18.25	63.50	1,630	49.1	[2]

[1]Carcasses graded by Mr. Richard L. Hiner, Meat Laboratory, Division of Animal Husbandry, Bureau of Animal Industry.
[2]Not graded; the quality did not cover a wide range, but the carcass of lamb 243 was definitely inferior to the carcasses of the other 3 lambs.

SYMPTOMS AND PATHOLOGY

Symptoms.—Application of infective larvae to the skin of lambs caused local inflammation and irritation. Penetration obviously caused immediately intense itching, for as soon as a lamb was released from restraint it would vigorously scratch with a hind foot the area to which larvae had just been applied. This tendency to scratch the skin sometimes persisted for several days. It was not determined to what extent the inflammatory reactions which followed application of larvae were due to response to the presence of the larvae, to

TABLE 7—Larvae Administered and Worms Recovered at Necropsy of Lambs in Experiment 2

Group No.	Lamb No.	Dates larvae were administered	Method of administration	Hookworm larvae administered: On indicated date	Hookworm larvae administered: Total larvae	Date of slaughter	Interval from start of experiment to date of slaughter	Worms recovered at necropsy: Strongyloides papillosus	Nematodirus sp.	Cooperia sp.	Trichuris sp.	Hookworms (*Bunostomum trigonocephalum*): Males	Hookworms (*Bunostomum trigonocephalum*): Females	Hookworms (*Bunostomum trigonocephalum*): Total	Relation of hookworms recovered to larvae administered[1]
		1943		No.	No.		Days	No.	No.	No.	No.	No.	No.	No.	Per cent
4	226	10-29	on skin	400,000											
		11-2		180,000	580,000[2]	3-18-44	141	2,318	0	17	7	50	33	88	0.0152
4	238	10-29	on skin	100,000											
		10-30		85,800											
		11-2		50,000											
		11-5		51,850											
		11-8		75,000											
		11-12		20,000											
		11-18		17,600											
		11-20		23,800											
		11-23		44,300											
		11-25		68,400											
		11-29		22,850	559,600[3]	3-17-44	140	2,128	24	57	5	95	100	195	0.0345
4	243	10-29	on skin	20,000											
		10-30		17,150											
		11-2		10,000											
		11-5		10,400											
		11-8		15,000											
		11-12		4,000											
		11-18		3,500											
		11-20		4,750											
		11-23		8,850											
		11-25		13,700											
		11-29		4,550	111,900[4]	3-17-44	140	2	3	98	4	1,008	968	1,976	1.7700
5	249	[5]	.....	.....	.....	3-18-44	141	276	13	27	2	1[6]	0	1[6]	.....

[1]By skin only.

[2]A total of about 30,000 Strongyloides larvae also administered.

[3]A total of about 209,000 Strongyloides larvae also administered.

[4]A total of about 41,800 Strongyloides larvae also administered.

[5]Control lamb; no larvae administered.

[6]Recovered in screenings from stomach; though care was taken to prevent such an occurrence, this worm probably had adhered to the screen just previously used to examine the small intestine of lamb 226 and very likely it did not actually come from lamb 249.

the introduction into the skin of bacteria, or to mechanical damage by scratching. Apparently, long-continued application of larvae to the same skin area was instrumental in causing the skin to become thickened and leathery in appearance.

Infected lambs showed no elevation of temperature, no inappetence and no diarrhea. The 2 heavily infected lambs (246, 243) showed clinical evidence of anemia, the skin and conjunctiva becoming noticeably pale, beginning about ten weeks after infection. Also, by this time these 2 lambs were in obviously poorer flesh than the un-

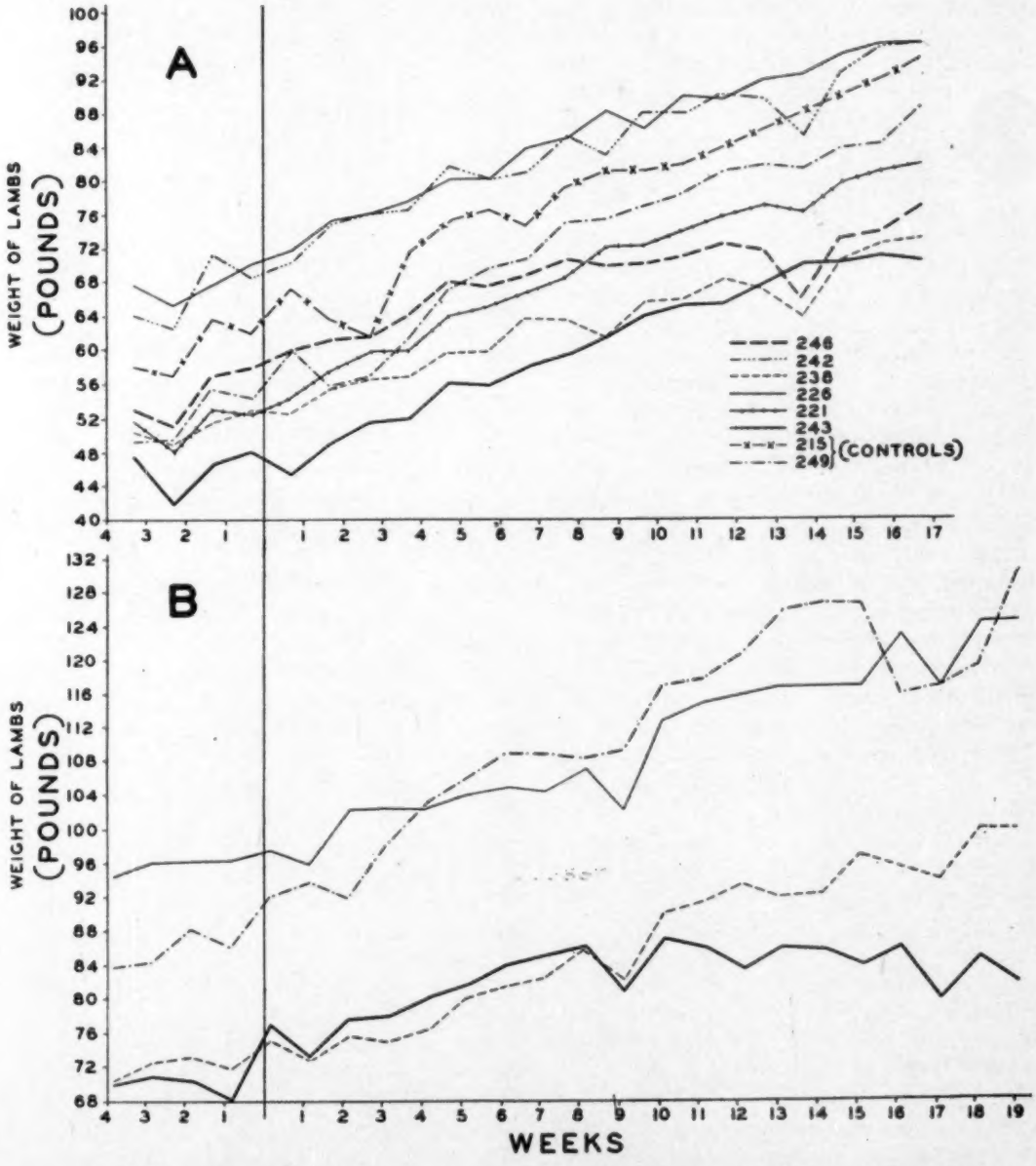

Fig. 4, A-B—Weekly weights of lambs in experiments 1 and 2 during each experiment and at the 4 weighings immediately preceding the first administration of larvae (indicated in each graph by the long vertical line denoting the 0-point on the time-scale) in each experiment. A—Weekly weights of lambs in experiment 1. B—Weekly weights of lambs in experiment 2.

infected controls. Lamb 246 appeared late in experiment 1 to have somewhat sunken flanks and a noticeable, though not prominent, degree of "pot-belly." Edema of the lower jaw was not observed. Thus, the only clinical evidences of infection were those indicative of anemia and failure to gain weight properly and only the more heavily infected lambs exhibited them.

Gross Pathology.—The abomasum, cecum and large intestine in all of the lambs were of normal appearance and were free of lesions. Small, hemorrhagic lesions, about 1 mm. in diameter, occurred in the small intestine in all infected lambs. These lesions obviously marked recent sites of attachments of the worms; they were identical in appearance to those observed when hookworms were detached from the mucosa. It was estimated that more such lesions were present in the small intestines of lambs 242 and 238 than in the intestines of lambs 246 and 243; this suggests that when fewer worms were present migration and reattachment were more frequent. The first 10 to 15 ft. and the last 30 ft. of the small intestine, even in the heavily infected lambs, were free of worms and lesions; in the intervening region, both worms and lesions were well distributed, but tended to be more numerous anteriorly. Some lesions were visible through the wall of the unopened intestine. Evidence of bleeding into the lumen was not observed. All other organs were of normal appearance in all lambs except that in lambs 246 and 243 they were slightly pale in color. There were no significant differences in the size and weight of organs (heart, lungs, liver, and spleen) among the lambs, except that in experiment 2 in lambs 243 and 238 the spleen weighed only about one-half as much as in lambs 226 and 249. In experiment 1, lamb 246 had markedly less omental fat than lamb 215 and in experiment 2, the omentum of lamb 249 was much heavier than that of any of the 3 infected lambs (table 6). No difference in the amount of fluid in the peritoneal or pericardial cavities was noted among the infected and control lambs.

TABLE 8—Results of Examinations of Feces of Lambs in Experiment 2 for Helminth Eggs and Coccidial Oöcysts

Group No.	Lamb No.	Bunostomum eggs per Gm. of feces found on examination carried out on indicated day after start of experiment									Average number Bunostomum eggs per gram[1]	Average number of Strongyloides eggs per gram[2]	Index of average density of oöcysts[3]
		3	10	17	31	45	59	73	87	101	115	129			
		No.	No.	No.	No.	No.	No.	No.	No.	No.	No.	No.			
4	226	0	0	0	0	0	4	42	124	530	132	66	149.67	4,019.90	0.50
	243	6	6	2	0	4	2,200	6,280	10,600	17,800	8,000	6,500	4,672.55	115.64	0.82
	238	2,000	700	800	400	264	1,200	265	580	700	200	400	682.64	1,903.00	0.50
Av.		...	...	...	...	...	...	...	...	...	...	...	1,834.62	2,012.85	0.61
5	249	0	0	0	0	0	0	0	0	0	0	0	0	375.20	0.75

[1]For infected lamb 226, average from time of first positive finding.
[2]Average of 11 observations carried out simultaneously with Bunostomum egg counts.
[3]Average of 11 density estimates carried out simultaneously with Bunostomum egg counts; for significance of numerical expressions of density, see footnote 4, table 2.

TABLE 9—Summary of Total Leucocyte Counts on Blood of Lambs in Experiment 2 and Proportions of Five Types of Leucocytes Counted Differentially in Blood Smears

Group No.	Lamb No.	Total leucocytes per cmm. of blood: In count in week before infection of test lambs	Total leucocytes per cmm. of blood: Average and extremes[1] in 12 counts usually at biweekly intervals	Average and extremes[1] of percentages of indicated types of leucocytes and of smudges in 11 counts usually at biweekly intervals: Lymphocytes	Neutrophiles	Eosinophiles	Monocytes	Basophiles	Smudges
		Thousands	Thousands	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent
	226	8.50	7.53 (4.65-10.26)	63.87 (48.00-73.40)	29.36 (20.20-48.60)	2.38 (0.20-5.40)	1.58 (0.80-2.80)	1.82 (0-3.80)	0.98 (0-2.00)
4	243	7.70	6.17 (4.05-9.05)	70.82 (60.40-84.00)	19.62 (11.60-24.40)	5.91 (0.40-14.60)	2.24 (0.40-4.60)	0.53 (0-1.40)	0.91 (0-2.40)
	238	5.25	5.26 (3.80-6.06)	78.74 (71.73-85.10)	15.34 (8.20-21.76)	2.87 (0.19-10.00)	1.43 (0.38-2.31)	0.81 (0.20-2.50)	0.81 (0-1.80)
Av.	...	7.15	6.32						
5	249	7.00	6.11 (4.60-8.50)	67.93 (55.20-74.80)	28.44 (22.80-41.20)	0.49 (0-1.00)	0.91 (0.20-1.40)	0.51 (0-1.20)	1.71 (0.80-2.80)

[1]Indicated by figures in parentheses.

DISCUSSION OF RESULTS

B. trigonocephalum has been regarded by authors as pathogenic for the following reasons: (1) its relationship to other hookworms of proved pathogenicity; (2) worms of this species appear to contain blood and to be blood-suckers; (3) the blood of infected sheep has been found to contain hemolysins; (4) hemorrhagic lesions occur in association with the attachment of this hookworm to the intestinal mucosa; and (5) in field studies of disease outbreaks, diagnosed as bunostomiasis, anemia, nervous manifestations, cachexia, and death have been observed. Observations on experimentally infected sheep, or sheep thought to have been so infected, have been very limited.

Our experiments show that heavy *B. trigonocephalum* infections caused anemia in lambs and indicated that these infections adversely affected the gain of growing lambs. To this extent, the views of various observers have been confirmed and the pathogenicity of the species has been established by experiment.

The anemia of the 2 most severely affected lambs was normocytic, but tended to be hypochromic. Landsberg[5] found acute fatal hookworm anemia in dogs to be normocytic, but Foster and Landsberg[6] found the anemia of non-fatal canine infections to be microcytic and hypochromic.

Serious disease and fatalities have been attributed to Bunostomum infections which were light compared to some induced in these experiments. Yet, our experimental lambs which at autopsy harbored from a few to about 500 hookworms had shown during their infection history no evidence of disease or at most only a slight anemia.

Beller[1] examined at autopsy, in the course of his investigation in the field, 40 sheep, presumably ones that had died or were about to die; he stated that hookworms only were found in the small intestine, but the numbers were not reported. He did report that when some of the animals that survived the disease outbreak were slaughtered later, because of deficient development, they no longer harbored any hookworms. Large numbers of *Nemato-*

dirus filicollis and *Chabertia ovina* were found in these lambs and Beller stated that had not the earlier diagnosis of hookworm disease been so definitely proved, the observed symptoms might wrongly have been attributed to these species. Later, Beller[2] performed an unconvincing experiment, as has been previously mentioned, and stated that bunostomiasis was the cause of death by cachexia of a yearling sheep given only 100 larvae and harboring at autopsy only 42 hookworms, some of which were immature; he concluded that relatively small numbers of hookworms can cause disease in sheep. Velu and Balozet[12] regarded bunostomiasis as the cause of death in flocks in which some lambs harbored at necropsy less than 100 hookworms. Although they stated also that a large number were frequently found in affected sheep, they did not define the size of these heavier infections more exactly.

Ortlepp[10] reported that a lamb which died nine weeks after it had been experimentally infected harbored about 200 nearly mature Bunostomum, was very anemic, and showed degenerative changes of the liver and marked gelatinization of the fatty tissues. He did not actually state that the hookworms were the cause of death or that the lamb was free of other parasites. It may not be correct, therefore, to infer that Ortlepp regarded bunostomiasis as the cause of death, but the language of the report suggests that he held this opinion.

In connection with these reports and our findings, two related questions arise. The first is whether farm or range sheep ever harbor as many hookworms as were recovered from the 2 experimental lambs that showed rather marked anemia. So far as we have ascertained, naturally acquired infections of similar magnitude have not been reported. In a series of 129 necropsies of sheep in Ontario, Fallis[5] found an average of 16 hookworms per infected sheep; the maximum number found in 1 sheep was 154. Cameron[3] found even smaller numbers in 38 infected, but healthy, sheep examined in Scotland. Curtice[4] stated that not more than 200 to 300 ever seem to be present in 1 sheep. Threlkeld[11], however, reported finding 494 hookworms in a yearling sheep in Virginia and more recently Habermann[7] has reported one infection of 634 and another of 982 hookworms encountered in the course of postmortem examinations of sheep reared at the Beltsville Research Center, Beltsville, Md. The 2 last-mentioned sheep had been kept under somewhat unusual conditions. They had been for a time in a bare feedlot containing a deep litter and later were transferred to small, overstocked pasture lots in certain parts of which litter had accumulated as a result of the necessity for supplementary feeding. Still these infections were far heavier than the average found in other animals kept on the same or similar areas at the same time or in other years, indicating that unusually heavy infections are acquired if favorable conditions obtain.

The second question is whether our experimental findings are inconsistent with reports of serious and fatal effects from comparatively light infections. With regard to experimentally induced infections of a few hundred or less hookworms in well cared for and well fed lambs or sheep, which originally were healthy, it can only be repeated that such infections were not found to cause noteworthy injury.

However, this finding is not regarded as necessarily inconsistent with the view that comparatively light, naturally acquired infections may sometimes be attended by serious consequences. There are two main reasons for taking this position. The first relates to the fact that opinion as to the seriousness of light infections is based largely on the findings noted at necropsy of sheep dying in the field. However excellently such findings may portray the picture at the moment, they may give little clue to the previous history of exposure to infection or to the size of the infection harbored earlier. Secondly, it seems well established that the severity of hookworm disease and anemia in man may depend upon several factors other than the number of worms harbored. Diet has been reported to be very important among these contributory factors.

The good diet fed our experimental lambs

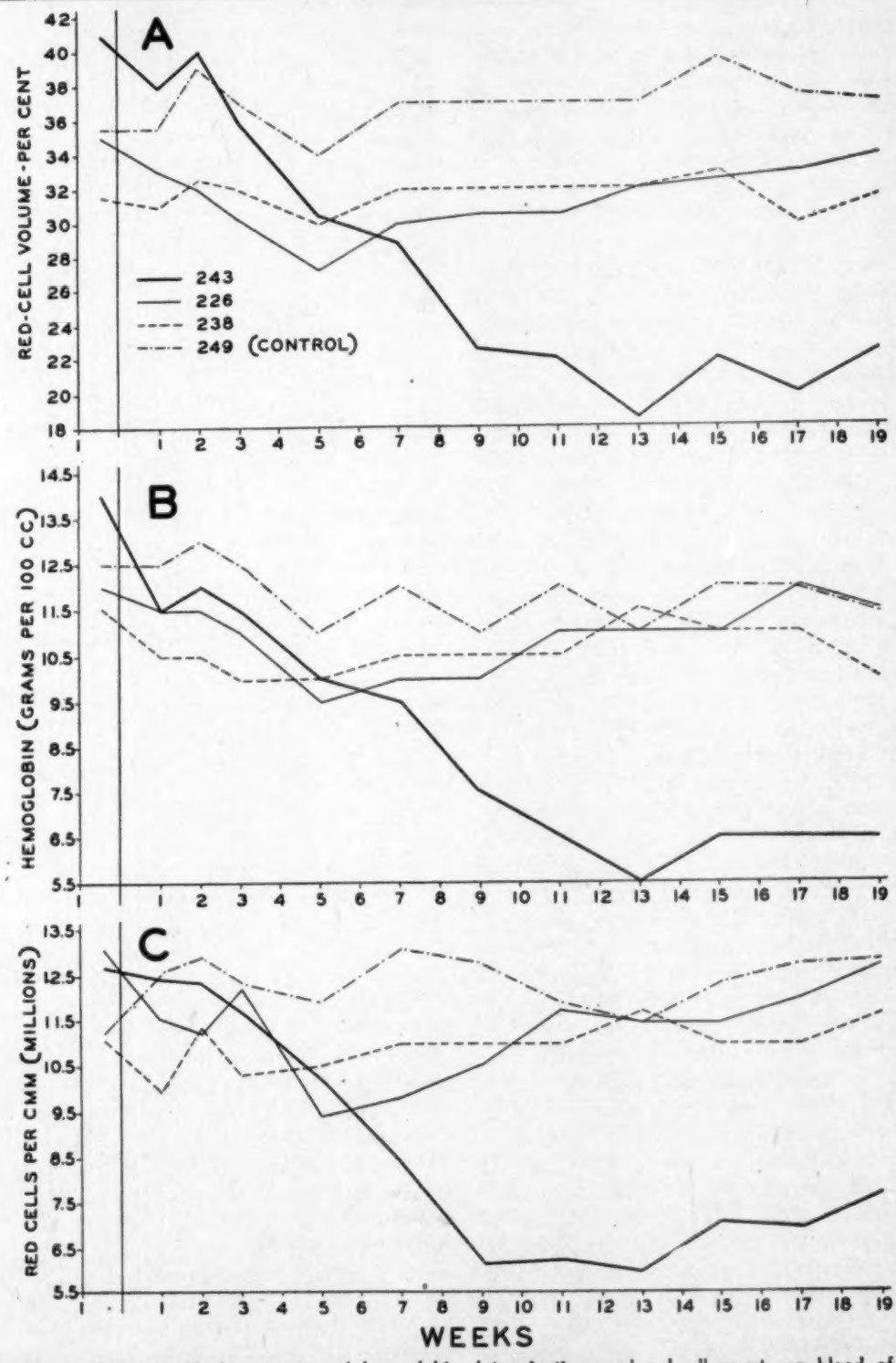

Fig. 5, A-C—Weekly hematocrit and hemoglobin determinations and red-cell counts on blood of lambs in experiment 2. (In each graph the long vertical line denotes the 0-point on the time-scale or initiation of the experiment by administration of larvae to the test lambs).

may have been important both in limiting the severity of the effects of the worms harbored by the test lambs and in favoring the recovery of the 2 most heavily infected ones from their anemia. Also, the animals were well housed and demands on their energy were extremely limited.

SUMMARY AND CONCLUSIONS

Two experiments to test the pathogenicity of *Bunostomum trigonocephalum* were performed at the Beltsville Research Center, Beltsville, Md., in 1943 and 1944. The object was to produce heavy experimental infections with this hookworm in lambs and to ascertain the effects produced thereby. Eight male lambs of mixed breeding were used in the investigation; they were about 4 to 4½ months old when the first experiment began.

B. trigonocephalum was found to be pathogenic. Heavy infections with this hookworm caused lambs to become anemic and depressed their rate of growth.

A lamb infected with 50,000 larvae percutaneously in one application harbored about 1,500 hookworms about four months later. When about 8 1/2 months old, a lamb that had a very light orally acquired experimental infection was exposed to further infection by about 110,000 larvae applied to its skin in 11 apportionments of moderate size, distributed over a period of one month; it harbored about 2,000 hookworms about four months after it first was infected percutaneously. Naturally acquired infections of these magnitudes have not been reported. The degree of anemia produced in these two lambs was moderately severe; in each, red cell values declined to about one-half normal levels, as observed in control lambs.

A lamb that received on its skin one application of 5,000 larvae harbored about 500 hookworms about four months later; it became slightly anemic, but its health was not otherwise demonstrably affected.

The maximum effects caused by still lighter infections were slight and very temporary anemia, and temporary interference with growth.

When 5,000 or 50,000 larvae were given orally, far lighter infections resulted than when these numbers of larvae were applied to the skin.

However, exposure of lambs to percutaneous infection by extremely large numbers (560,000 to 580,000) of larvae apparently provoked processes, of undetermined nature, which were highly effective in inhibiting the development of adult worms. In view of the previous history of infection of the 2 lambs so exposed, the results obtained, and other data of the experiments, it was concluded that neither acquired nor age resistance can be regarded as adequately explaining the relatively light infections produced by these large larval administrations.

No evidence was obtained that Bunostomum infection affected the total numbers of leucocytes in the circulating blood. Differential counts showed, however, that all significantly infected lambs had a degree of eosinophilia related directly to the magnitude of their hookworm burdens. Such infections also tended to cause slight monocytosis and basophilia.

In each experiment, the degree of anemia observed in significantly infected lambs tended to be directly related to the magnitude of the per-gram counts of hookworm eggs passing in their feces and to the magnitude of their hookworm burdens.

The anemia of the heavily infected lambs was normocytic, but tended to be hypochromic.

The presence of occult blood in the feces of the more heavily infected lambs was readily demonstrated beginning about twenty-five days after infection. The estimated comparative intensity of the benzidine-hemoglobin color reactions given by the feces of infected lambs was positively correlated with the observed degree of their anemia.

The prepatent period of *B. trigonocephalum* in moderate to heavy infections was found to be between fifty-three and sixty days.

Irritation of the skin followed penetration by hookworm larvae. At necropsy, the only gross evidences of injury due to in-

fections were hemorrhagic lesions in the small intestine caused by the attachment of the worms and the pale color of organs and tissues in heavily infected lambs. The carcasses of the 2 heavily infected lambs were of relatively poor quality.

Anemia and malnutrition in heavily infected lambs were the only observed symptoms of infection among the several ascribed to bunostomiasis by other investigators. However, this circumstance, as well as the fact that even the heavily infected lambs had begun to recover from their anemia within three and a half to four months after infection, may have been due to the good housing, care, and feeding received by the test animals.

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Corrigenda

In the January, 1946, issue (p. 28) of the AMERICAN JOURNAL OF VETERINARY RESEARCH, the sub-head should read "Results of *Injections* with Heated Serum-Virus Mixtures."

On pages 38 and 39, the graphs should be interchanged, the legends remaining in their original positions.

The Transmission of Lymphomatosis in the Fowl

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IT IS generally agreed that the incidence of lymphomatosis* in different strains of fowl depends upon (1) the pathogenicity of the causative agent, (2) the degree of exposure to the causative agent, and (3) the genetic susceptibility of the host to the disease.

The value of increasing the exposure to lymphomatosis in order to select more accurately those families which are resistant to the disease has been discussed by DeOme.[1] Great changes in the genetic resistance to lymphomatosis cannot be attained quickly in most good strains of chickens where some previous selection for viability has been practiced. When mortality reaches relatively low levels, it becomes difficult to further increase the resistance to disease. However, further selection for resistance may be desirable if some chicks from the flock are likely to encounter much greater exposure to the organisms of the disease than the environment of the breeding farm affords. For most effective selection, we may assume that a level of mortality between 30 and 70 per cent is desirable.[4] Some technique for uniformly increasing the exposure to lymphomatosis is needed if such a high incidence is to be attained in breeding flocks where selection for resistance has been made during several years. Artificial infection with the causative agent is one means of increasing the incidence of a disease. However, investigators have experienced difficulty in transmitting lymphomatosis.[10]

Reports of the transmission of lymphomatosis have been made by Patterson *et al.*,[13] Johnson,[5] Jungherr,[6] and Lee *et al.*,[8] but the results in the hosts were complicated by blood forms of the avian leucosis complex and characterized by long incubation periods. For most effective selection, the transmitted disease should duplicate the field cases except with respect to the incubation period, which should be shorter. The experience, in this locale at least, has been that the great majority of birds that come to autopsy with lymphomatosis are free of blood dyscrasia. Over a period of years, during which more than 10,000 autopsies were made, there were no cases of erythroid or myeloid leucosis noted by the writers. That these forms of leucosis are rare in this area is corroborated by DeOme.[2] The successful transmission of lymphomas uncomplicated by blood dyscrasia and with a relatively short incubation period was reported by Olson.[11]

There are two possible interpretations in the transmission of the tumor cells. Diseased cells which proliferate in the medium of the new host may be transplanted, or an agent closely affiliated with the tumor cell may incite previously normal tissue to tumefaction. The panornithic proportions that lymphomatosis has assumed have led to the probably correct general assumption that the disease is transmitted by an agent, although no specific agent has as yet been identified. Olson may have transmitted the causative agent of lymphomatosis along with lymphomatous tissue, but no evidence has been presented to support that hypothesis.

The present investigation was undertaken to study a procedure for increasing mortality from a type of lymphomatosis identical with, or similar to, that which occurs from natural exposure to the disease. It provides additional evidence that the tissue used for transmitting the disease actually carried the agent which causes spontaneous lymphomatosis under conditions of natural exposure.

From the Kimber Poultry Breeding Farm, Niles, California.

*In this study, lymphomatosis is defined as aggregates of lymphomatous tissue large enough to be visible on gross inspection and uncomplicated by blood dyscrasia.

MATERIALS AND METHODS

Infective material for inoculating chicks was obtained originally from several birds of a large flock of Single Comb White Leghorns. Tissue from some lymphomatous livers and spleens, when inoculated into chicks, resulted in high mortality at an early age, and with lesions typical of spontaneous visceral lymphomatosis in this flock.

One strain of the infective material was selected for continued study. The original donor of this strain (a hen weighing 1,465 Gm.) was destroyed at 407 days of age. The pupil of the right eye was irregular in outline, eccentric, and the surrounding iris was slightly grey. At autopsy the liver weighed 220 Gm., the spleen weighed over 20 Gm., and both organs presented lymphomas. This strain was maintained by serial passage through day-old chicks from the same flock in which the donor was found.

Infective material was standardized after the first transfer. Moribund birds were selected, antemortem blood smears were made, the birds were destroyed by dislocation of the neck, and the body cavities were opened under aseptic conditions. The spleen and most of the liver of the suitable birds were removed and ground with a pestle in a mortar lined with a wire screen. Enough 0.85 per cent saline was added to produce a dilution of 1 part tissue to 25 parts diluent, and the mixture was strained through muslin. All equipment was sterilized before use. Sections of liver were placed in 10 per cent formalin solution.

For oral administration, lymphomatous tissue from liver and spleen was prepared in the same way, except that it was diluted with equal parts of saline.

Inoculation of chicks was done on the second day after hatching, which was the twenty-third day after the beginning of incubation. The material of the original donor was injected intraperitoneally with a 22-gauge needle, about 0.25 cc. per chick. All subsequent transfers were made with a 26-gauge needle subcutaneously. Beginning with the eleventh passage, the volume injected was reduced to 0.10 to 0.15 cc. per chick.

Feeding of the lymphomatous tissue to chicks was done with a special pipette made to deliver about 1 cc. of the prepared tissue into the crop of day-old chicks. At the same time that lymphomatous tissue was fed to the chicks, 1 drop of the mixture was placed in the left eye and 1 drop placed over the left nostril. This procedure was followed to provide the mucous membranes of the body with a high degree of exposure to the inoculum.

The hosts were White Leghorn chicks from the same strain in which donors were found. The chicks were themselves individually pedigreed, and were the progeny of parents whose sibs' viability had been relatively high.

Beginning with the eleventh passage, the hosts were divided into three groups. The first group was inoculated. The second group (contacts) was brooded with the inoculated sibs. The third group of sibs was brooded in separate pens not adjacent to the pens containing

TABLE 1—Mortality Among Inoculated, Contact, and Control White Leghorn Sibs, 1943

Age in Days	Inoculated 1215 chicks		"Contacts" 797 chicks		Controls 1923 chicks	
	Dead from		Dead from		Dead from	
	All causes	Lympho-*	All causes	Lympho-*	All causes	Lympho-*
	(per cent)	(per cent)	(per cent)	(per cent)	(per cent)	(per cent)
10 to 100	76.2	59.8	3.2	0	5.4	0.05
10 to 200	Discontinued		6.5	0.9	7.6	0.2
10 to 300			12.6	3.4	10.3	1.4
10 to 400			19.3	5.0	13.6	2.2

*Dead with gross lymphomatous lesions.

TABLE 2—Birds (10 to 100 Days Old) Dead with Lymphomatous Lesions in Group Fed Lymphomatous Tissue and in Control Sibs from the Same Hatches, 1943

Date of Hatch	Fed Lymphomatous Tissue		Controls	
	Chicks	Dead with Lymphomas 10-100 days of age	Chicks	Dead with Lymphomas 10-100 days of age
	(Number)	(per cent)	(Number)	(per cent)
May 6, 1943	537	7.6	227	0.9
May 13, 1943	515	9.3	273	0.0
May 20, 1943	516	3.7	261	0.0

the inoculated and contact birds, and was considered as a control group.

The chicks to which lymphomatous tissue was fed came from three hatches of the 1943 season. Sibs from these same hatches were used as controls. The control chicks were brooded in pens not adjacent to "fed" or inoculated birds.

The groups of birds inoculated or fed could not be kept intact for longer than 100 days of age with available facilities.

Diagnoses of the causes of death were made without reference to experimental treatment in order to avoid bias. Autopsies were performed on large numbers of other birds at the same time. For this study all chicks that died before they were 10 days of age were eliminated from the data because it was presumed that most of the deaths during the first few days after hatching resulted from causes other than tumor growths. Some cases of death with lymphomas did occur before 10 days of age, but these were few, and the lesions were usually too indistinct to have been included in this report. Only macroscopic lymphomas were considered in this report, although it is recognized that death from lymphomas might take place before adequate time had elapsed for the development of gross lesions.

Some histologic sections were studied. Liver slices, previously placed in 10 per cent formalin, were transferred to 40 per cent formalin for one hour and then sectioned on the freezing microtome into 20 μ thick slices and stained by the hematoxylin-eosinol method described by Krajian.[7] Blood smears, taken antemortem from all prospective donors were stained with Wright's stain and studied for any evidence of blood dyscrasia. Tissue impressions (made by impressing a fresh cut section of the liver on a slide and staining with Wright's stain) were made on some birds and were studied for evidence of blood dyscrasia.

RESULTS

In the inoculated birds, lymphomas were found in the liver, spleen, kidneys, lungs, heart muscle, pancreas, various portions of the gastrointestinal tract and mesentery, the adrenals, testes, ovary, periocular tissue, skin, and muscle. Liver lesions typical of those found in a majority of young inoculated chicks are shown in figure 1.

Fig. 1—Typical lymphomatous lesions in the livers of chicks inoculated with liver and spleen tissue from donors with similar lesions.

Tumors in the skin and muscle were infrequent except at the site of inoculation. At the site of inoculation, lymphomas were not constant even though they were present in other parts of the bird. The histologic picture of the tumors found in the hosts was monotonously uniform and was similar to that seen in the great majority of tumors found in the birds of the flock from which the donor came. They were characterized by large, round, lymphoid cells. There was no evidence of blood dyscrasia in blood smears or in tissue impressions.

Of 2,197 inoculated chicks in these studies that were alive at 9 days of age, 1,275 (58%) had died before 100 days of age

with lymphomas visible on gross inspection. Among the contacts no lymphomatous lesions were found in the chicks that died during the first one hundred days of life. Among the controls, 1 chick was recorded dead with lymphomas at 11 days of age. This was probably an error.

In the first passage, the first bird showing lymphomas died thirty days after it was inoculated. Among 125 hosts in this first passage, 48 per cent presented gross lymphomatous lesions at death within one hundred days after the date of inoculation. As the number of passages increased, the course of the disease became more rapid. This is shown in figure 2 by the earlier initial death with lymphomatous lesions, the younger age at which 50 per cent of the chicks were dead showing lymphomatous lesions, and the younger age at which 50 per cent of the chicks were dead from all causes. During the later passages, the 50 per cent mortality levels were reached at ages less than half of those observed during the first passage.

The mortality among the three groups of sibs hatched together in 1943 is shown in table 1. The relatively high incidence of lymphomatous lesions among the inoculated birds suggests that it may be feasible to obtain an increased incidence of typical lymphomatosis for experimental study. There are two possible explanations for this high incidence of typical lesions. One may reason from such results that malignant cells have merely been transmitted from donor to host, that multiplication of these cells should be expected to give typical lesions. Such results provide no evidence that a causative agent of lymphomatosis was involved in the passages described.

However, the chicks brooded in contact with the inoculated birds provide convincing evidence that the infective tissues used for inoculation actually carried the causative agent of lymphomatosis. Because the contact chicks received no transplants of malignant cells, the mortality from lymphomatosis should have been comparable to that among controls, unless the inoculated chicks carried abnormally large amounts of the causative agent of lymphomatosis.

Actually, the mortality from lymphomatosis to 400 days of age was nearly twice as great among the chicks brooded with the inoculated birds as it was among controls. This difference is highly significant (value for $P < 0.01$). There is no apparent difference in the environmental exposure of the contact and control chicks studied except the association of the contact chicks with inoculated birds. Since it can be assumed that the inoculated birds could not transmit something they did not possess, it seems clear that following inoculation they must have carried unusually large amounts of the causative agent of lymphomatosis.

The experiments on feeding lymphomatous tissue were initiated because it was realized that parenteral inoculation transgresses natural defense mechanisms afforded by the skin and mucous membranes. Also, if the inoculum used in these experiments did contain the agent of lymphomatosis, then administration of the inoculum to the mucous membranes should have resulted in lesions typical of lymphomatosis even were malignant cells less likely to enter the vascular system. If the mucous membranes constitute a natural defense mechanism, then a decreased incidence of the disease in relation to inoculated birds should be expected, although an increased incidence in relation to control birds should be expected. Furthermore, if oral administration should prove to be the natural route of infection, it is likely that a more accurate measure of differences in resistance could have been obtained by feeding family representatives than by inoculating them.

There were 1,568 chicks fed lymphomatous tissue. Of these birds, 6.9 per cent died with lymphomatous lesions before they were 100 days of age. At autopsy, it was impossible to distinguish by the type or distribution of lesions whether a chick had been fed, or had been parenterally inoculated, with lymphomatous tissue. A control group of 761 sibs was hatched with the fed chicks. Only 0.3 per cent of these chicks died before 100 days of age with lesions of lymphomatosis.

The difference in mortality between birds

fed lymphomatous tissue and the control sibs is significant. Furthermore, when the data from different hatches are considered separately, the difference between the fed and control chicks is found to be consistent, and highly significant for each hatch (table 2).

There is a possibility that virulent lymphomatous cells might attach to a portion of the exposed mucous membrane, replace some of the normal tissue by tumefaction, and metastasize from there to other parts of the body. However, considered in conjunction with the evidence submitted by the contact birds, the higher mortality of the fed chicks, when compared to the control sibs, supports the hypothesis that the lymphomatosis producing agent was present in the minced tissue fed.

DISCUSSION

Olson[11] succeeded in transmitting lymphomas from a field case by intramuscular injection with lymphomatous tissue. However, he did not claim to have transmitted an infective agent, presumably because his results might be explained by the transplantation of malignant cells.

Tissue from freshly collected lesions of visceral lymphomatosis would be expected to contain the infective agent of that disease. However, such difficulty has been met in transmitting the disease that Feldman[3] and others considered it to be nontransmissible.

The results of this study show that lymphomas have been readily transmitted to inoculated chicks. Furthermore, the chicks brooded in contact with the inocu-

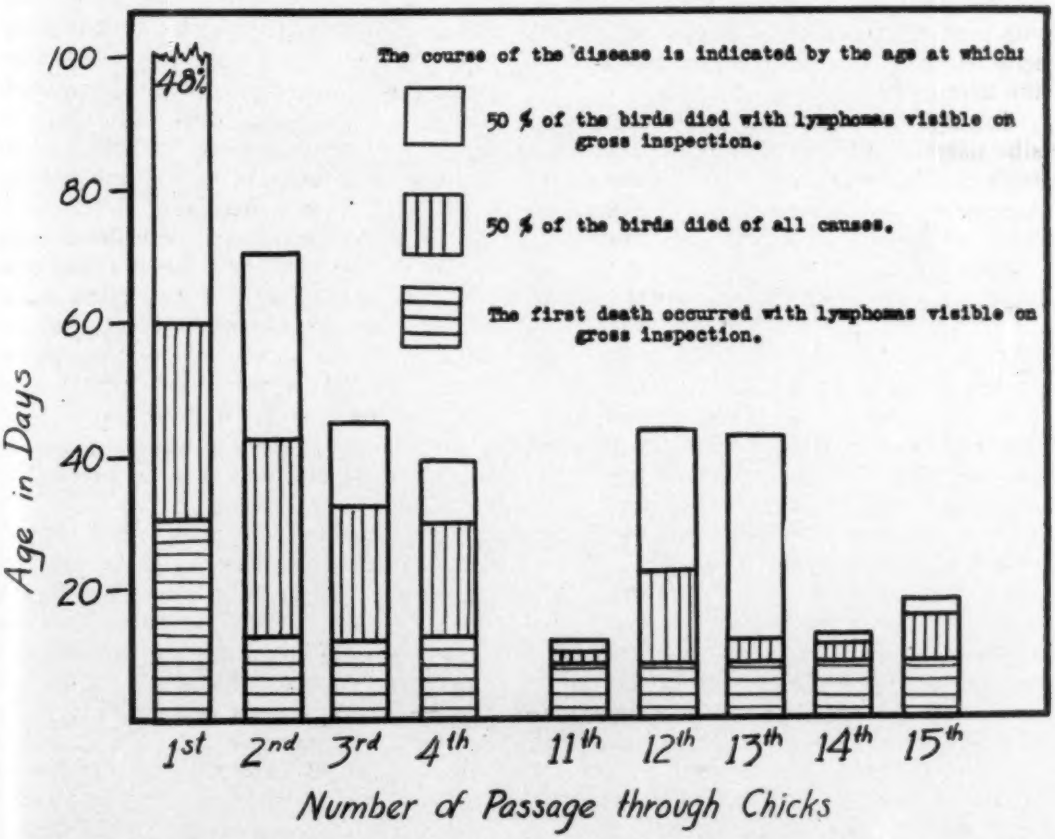

Fig. 2—The effect of serial passage on the course of a disease characterized by lymphomatous lesions.

lated birds suffered significantly higher mortality from lymphomatosis than did controls brooded in other pens. Therefore, the inoculated chicks must have carried unusually large amounts of the etiological agent of lymphomatosis in order to have so increased the incidence of this disease among contact chicks.

SUMMARY

Tissue from a lymphomatous liver and spleen from a typical field case of lymphomatosis (uncomplicated by blood dyscrasia) was transmitted into White Leghorn chicks through 15 passages.

In the fifteenth passage, 59 per cent of the birds died with lymphomatous lesions before they reached 100 days of age. Fifty per cent of these inoculated chicks were dead with lymphomatous lesions within twenty-four days.

Chicks raised in contact with (brooded with) sibs inoculated with the infective tissue developed a significantly higher incidence of lymphomatosis than did controls.

Chicks fed the infective tissue developed a significantly higher incidence of lymphomas than did the control sibs from the same hatches.

ACKNOWLEDGMENT

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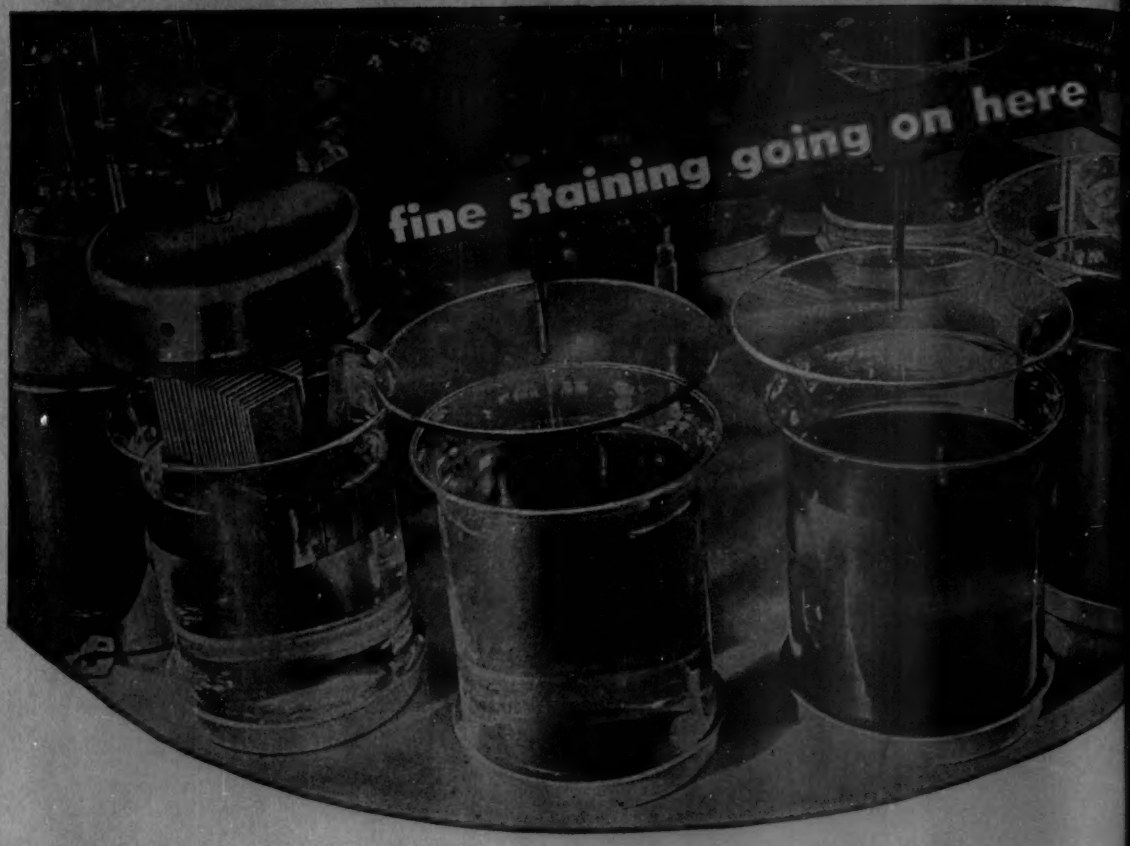

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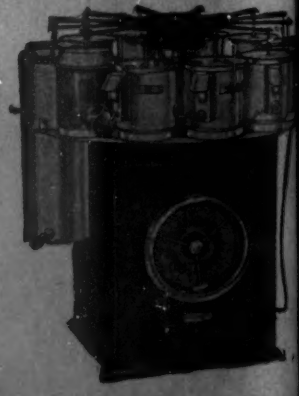

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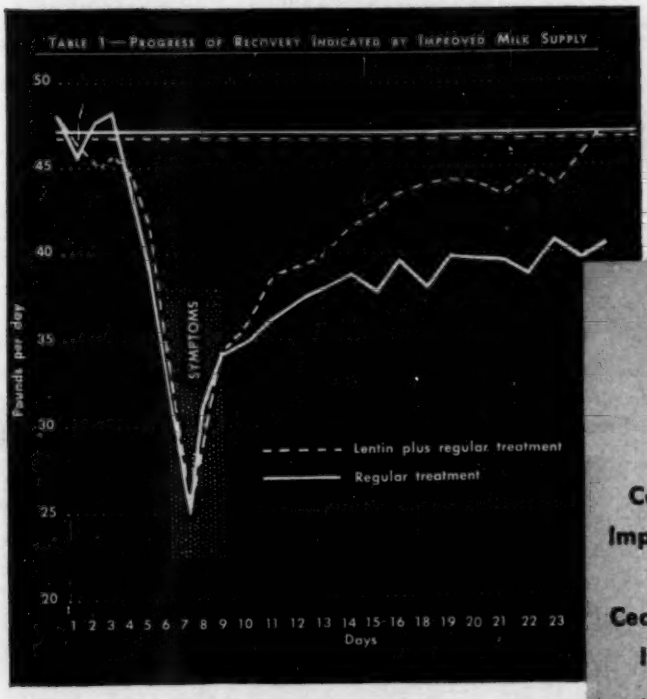

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In Two Parts — Part 2

Note

[*Part 1 of the April number of the* AMERICAN JOURNAL OF VETERINARY RESEARCH *constituted the regular issue. In Part 2 we are privileged to publish, by special arrangement with the Joint United States-Canadian Commission and the Chemical Warfare Service, U. S. Army, a series of manuscripts describing some of the important animal-disease research carried on during the war. The work reported in these papers was highly secret and the records of it were only recently released for publication. The purpose of the project was to develop ways and means of protecting the livestock industry of the North American continent against a foreign plague that might be introduced intentionally, as an enemy method of "biological warfare," or accidentally.*

Six officers of the U. S. Army Veterinary Corps, one from the Medical Corps of the U. S. Navy, and two Canadian scientists, together with a corps of technicians from both countries, were assigned to the project. The highly successful outcome of the work in developing protective measures against rinderpest, one of the most devastating diseases of cattle, including improved methods of vaccine production, plus fundamental observations significant to virus-disease research constitute an outstanding contribution to veterinary science and another shining example of what can be accomplished through collaboration of scientists from several fields.

In the July issue, several manuscripts dealing with another wartime research project will be published.—The Editors.]

Foreword

Rinderpest, the Preparation of Tissue and Egg Vaccines

The possibility of the introduction of rinderpest into this continent has long been a matter of serious concern. As a result both of increasing air travel and suspected enemy interest in biological warfare, this possibility became alarming in the latter part of 1941. Following various informal conferences, a Joint United States-Canadian Commission was appointed by the United States Secretary of War and the Canadian Minister of National Defense, with the following members:

J. CRAIGIE, Associate Professor of Immunology and Bacteriology, University of Toronto, Toronto, Ont., Can.
R. E. DYER, Assistant Surgeon General and Director, National Institute of Health, U. S. Public Health Service, Washington, D. C.
E. B. FRED, Professor of Bacteriology, and Dean, Graduate School, University of Wisconsin, Madison Wis. (Later Dean, College of Agriculture, and now President, University of Wisconsin.)
R. A. KELSER, Chief, Veterinary Division, Surgeon General's Office, War Department, Washington, D. C. (Now Dean, and Professor of Bacteriology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia.)
C. A. MITCHELL, Dominion Animal Pathologist, Animal Diseases Research Institute, Department of Agriculture, Hull, Que., Can.
E. G. D. MURRAY, Professor of Bacteriology, McGill University, Montreal, Que., Can.
G. B. REED, Professor of Bacteriology, Queen's University, Kingston, Ont., Can.
H. W. SCHOENING, In Charge, Pathological Division, Bureau of Animal Industry, U. S. Department of Agriculture, Washington, D. C.

Early in 1942, an island was acquired in the lower St. Lawrence River and a research and development laboratory established, with a joint United States-Canadian scientific staff and Canadian army service personnel. The relative inaccessibility of the location made possible the necessary isolation. Existing buildings were reconstructed to permit of rigid quarantine and enforcement of safety procedures. However, the Commission is more indebted to the scientific staff for its sound technique than to geography or equipment for the safe handling of large amounts of material and many experimental animals for a period of over four years.

The scientific staff was given two problems. The first was to prepare a tissue vaccine according to methods previously developed, in order to provide rapidly the means of surrounding an epizoötic, should it occur, with a ring of immunized animals. The success of this procedure is indicated in the third paper in the following series. The second problem was to investigate the possibility of developing an efficient vaccine which could be produced more economically and without requiring the use of large numbers of animals. Subsequent papers in the series detail the manner in which the virus was adapted to embryonated hens' eggs and attenuated for cattle with retention of high antigenic properties. The final papers in the series indicate the methods used in the large scale production and testing of the avianized vaccine.

In addition to monthly reports on the progress of the work, the scientific staff prepared papers in form for publication as each phase of the work reached a reasonably conclusive stage. These papers were deposited with the Commission. The following sixteen papers are now published without change and with the date of their receipt by the Commission shown in a footnote to each title.

The avianized vaccine is now being extensively tested in one of the countries where the disease is enzoötic. Reports on the results will be published when the work is completed. It may be stated, however, that the avianized virus has proved to be so attenuated that only mild and negligible reactions are produced in native cattle which are more highly susceptible to the disease than European breeds. At the same time, both native and European breeds are well protected by the vaccine against the virus strains from which the avianized virus was developed and against enzoötic virus strains.

The Commission wishes to congratulate the scientific staff on an arduous and brilliant performance under difficult circumstances and to commend the following Canadian and United States non-commissioned personnel for devotion to duty which made the work possible:

T/Sgt. T. R. James, S/Sgt. D. W. Dewar, WO II, T. Stovell, S/Sgt. R. P. Hanson, WO II, A. S. Greig, PhM2/c J. J. Humphrey, T/3 R. H. Shope, Sgt. P. B. McDonnell, S/Sgt. A. Rudacille.

Rinderpest

I. The Cultivation of Rinderpest Virus in the Developing Hen's Egg

COMMANDER RICHARD E. SHOPE, M.C., U.S.N.R.; CAPTAIN HENRY J. GRIFFITHS, General List, Canadian Army; CAPTAIN DUBOIS L. JENKINS, V.C., U. S. Army

RINDERPEST is a highly infectious, highly fatal disease of cattle, epizoötic or enzoötic in various areas of the Eastern Hemisphere but especially prevalent in parts of the Orient, in India, and in Africa. The disease has never occurred in Canada or the United States and, except at the Grosse Isle laboratory, is at the present time nonexistent in the Western Hemisphere.

The first War Disease Control Station was established at Grosse Isle, P. Q., Canada, for the purpose of studying rinderpest and developing adequate protective measures against it. The immediate objectives were two in number: first, we were to produce as rapidly as possible an adequate stock of virulent rinderpest virus, and, secondly, we were to convert this virus into a safe vaccine effective in protecting cattle against rinderpest.

Two chemically inactivated anti-rinderpest vaccines have been used successfully. One of these is the chloroform-inactivated vaccine of Kelser, Youngberg, and Topacio, and the other is the formalin-inactivated vaccine used extensively by Curasson and Delpy, Daubney, Pirani, and Jacotot. We proposed, at the outset, to prepare our vaccine after the method of Kelser and his coworkers, and the Commission recommended that we prepare, and have available at the earliest possible moment, at least 100,000 doses.

Both the chloroform- and formalin-inactivated vaccines used heretofore have been prepared from virus-infected tissues of cattle ill of rinderpest, and there are certain unfavorable features about such preparations. In the first place, the yield of vaccine from an individual bovine animal is not large and the titer of virus in the organs used in preparing the vaccine is not impressively high. Secondly, there is always the hazard of including bacterial pathogens dangerous for cattle in a vaccine prepared from bovine tissues. In order to circumvent the unfavorable features of the bovine tissue vaccine, we have tried to establish rinderpest virus in the developing hen's egg. It seemed possible, if the agent could be cultivated in this way, that a richer and more economical source of virus for vaccine preparation might be provided. Furthermore, under conditions of cultivation in embryonating eggs, the virus could be grown free of bacteria and the hazard of introducing bacterial pathogens with the vaccine would be avoided. The present report describes the methods that have been used and the results obtained in cultivating rinderpest virus on the chorioallantoic membrane of the developing hen's egg. The results obtained in using egg-cultivated rinderpest virus as the basis for an anti-rinderpest vaccine will be described in a subsequent report.

Received by the Commission on 30 July 1943 for publication which had to be delayed on account of security regulations in effect at that time.

EXPERIMENTAL

Source of Virus.—The rinderpest virus used in the present experiments was an African bovine strain. It came to us dried in sealed ampules, and examination of the powdered fragments indicated that the dried tissue was lung. No difficulty was encountered in establishing the virus in calves. Administered subcutaneously to calves, it induced a disease that was clinically characteristic of rinderpest, as described in textbooks, after an incubation period of from forty-eight to seventy-two

hours. Most of our animals have been sacrificed on the second or third day of fever, but those allowed to progress have died after from six to ten days of illness. The experimental disease is contagious between calves, transmitting by pen contact after incubation periods of six and eight days in the 2 experiments that have been conducted.

Experimental Animals.—We have used, for all work so far, calves purchased either locally or at the Montreal stockyards. These have ranged in age from 6 months to 1 year and we have purposely included representatives of as many breeds as could be procured. Most of the animals have been Holstein-Friesians, Jerseys, or Ayrshires, but a few Herefords, Red Polls, Shorthorns, Guernseys, and Canadians have been included. We have as yet used no Aberdeen-Angus or Brown Swiss. So far as can be told from experiments that have been largely qualitative, all of the breeds from which we have used representatives are of about equal susceptibility to our strain of rinderpest.

Isolation Technique and Precautions.—We have taken every precaution we could devise to prevent its escape from the laboratory and isolation quarters at Grosse Isle. Furthermore, in order to insure the accuracy of results obtained in individual experiments, it has been essential that isolation of individual experimental animals be rigid and absolute. Ten isolation units, constituting a modification of those used successfully at the Rockefeller Institute in Princeton, N. J., in working with swine influenza and hog cholera, were built. They consist of a main room in which the animal is kept and an anteroom, separated from the main room by a wall and door, in which is a shower bath for the attendant. Rubber gloves, boots, and coveralls are provided for the robing of the attendant while he is in the main room with an infected animal. These are removed and left in the main room when the attendant has completed his chores and passes into the shower-bath anteroom. The attendant takes a soapy shower between caring for animals in individual rooms. No bedding is provided for the animals, but the floors of the isolation rooms are of broom-finished concrete to prevent slipping. Manure is disposed of in the bowl of a "flushometer" type toilet located at one end of each isolation room. These toilets, as well as all other sewer lines from the isolation quarters, connect with a special type of steam sterilizing pit which will be described later. Animals in isolation are fed no hay, because remnants of this would be difficult to dispose of with the sewage facilities in use. Instead they are maintained on an adequate diet consisting of a commercial grain dairy ration mixed with dried beet pulp and ground alfalfa. On such a diet, individual animals have been maintained in good condition for periods as long as four months. At the termination of experiments, the animals are subjected to autopsy in the rooms in which they have been kept in isolation. For disposal, the carcasses are cut into pieces small enough to fit into large, metal garbage cans. These cans, when filled, are thoroughly scalded with very hot water before removal from the unit and transportation to the incinerator. Final disposal of the carcasses is by incineration. After removal of the carcass from an isolation room, the attendant thoroughly cleans the floor and walls with scalding water from a hose, removing adherent manure and debris with a coarse brush and scraper. Thermometers are sterilized by soaking in cresol solution, and boots, rubber gloves, and coveralls by soaking for an hour or longer in a closed garbage can of scalding water.

In addition to the ten individual isolation units, there is a larger production room planned similarly to the individual units, but much larger. This production room has a shower-bath anteroom like the individual isolation quarters just described, but its main room is large enough to contain 14 box stalls, arranged 7 on each side of a central corridor. At the end of the room containing the stalls is an autopsy room. Entrance to and exit from either of these rooms is only by way of the shower-bath anteroom. The isolation technique and precautions outlined for entering or leaving the individual isolation units are observed

and practiced in the production room. While the segregation of individual animals is not nearly so rigid in this room, we have found that, if separate thermometers, pails, and feed boxes are provided for each stall, and if reasonable care is exercised by the attendant, cross infections do not occur among the animals housed in these quarters. We have on several occasions placed normal, uninoculated calves in one of the stalls at a time when there were rinderpest-infected calves in the other stalls and no infection of the normal calves occurred, despite the fact that the attendant purposely fed, watered, and took the temperatures of the normal animals last each morning and evening. However, even though the evidence at hand indicates that individual box stalls provide adequate segregation to prevent cross infection, crucial experiments are conducted only in the individual isolation units and the production room is used, as its name implies, for the production of virus for vaccine or for the conduct of preliminary experiments.

The steam sterilizing pit to which all manure and sewage from all of the isolation quarters flows consists of two concrete tanks of 1,000-gal. capacity each. Coils of steam piping are arranged along all four sides of both tanks from the bottom to almost halfway up the side of the tank, and these coils are connected with the steam line from the boiler room. The two tanks are so arranged that only one at a time can receive sewage. When the one tank has been filled, the flow of sewage is diverted to the other tank and steam is then turned into the coils of the full tank. The sewage is heated until it has reached a temperature of 80 C. Then, after standing at this temperature for half an hour, it is pumped from the tank, by means of a London bellows pump, into a line leading to the river. Approximately four hours' heating with steam is required to bring the temperature of the sewage to 80 C. During approximately half this time, the temperature of the tank's contents is above the thermal inactivation point of rinderpest virus. When the second tank has been filled, its contents are heated as just described, and the flow of fresh sewage is diverted to the previously emptied tank. Under ordinary working conditions we have found that each tank suffices for about two days' sewage collection.

ATTEMPTS TO CULTIVATE RINDERPEST VIRUS ON THE CHORIOALLANTOIC MEMBRANES OF EMBRYONATING EGGS

Our initial attempts to cultivate rinderpest virus in embryonating hen's eggs were complicated by a series of failures of electrical power, during which both inoculated eggs and preinoculation eggs were maintained at temperatures as low as 22 C. for periods of as long as eighteen hours at a time. Furthermore, as an additional complication, the thermoregulator on the only incubator in which we could place infected eggs was, during the first six weeks of this work, incapable of adjustment to closer than 6 degrees. Thus, this incubator constantly and rather rhythmically fluctuated between 32 C. and 38 C. Because, in the first series of experiments carried out under these irregular conditions of incubation, we apparently succeeded in maintaining rinderpest virus for four serial passages in eggs, we toyed for a while with the view that perhaps our success was in some way accounted for by these temperature fluctuations. We have subsequently been able to demonstrate that this was not the case and that variations in temperature below 36 C. were actually deleterious to prolonged maintenance of the virus in fully virulent form in eggs. Eventually, our electric generators were got into a more dependable state of performance, some small bacteriologic incubators capable of accurate temperature adjustment were obtained, and we were able to proceed in attempting to account for variations in the initial, apparently positive results we had obtained. Before these things happened, however, we carried out approximately six weeks of work during which three separate sets of egg cultures were initiated.

Technique of Cultivation of Rinderpest Virus in Eggs.—Fertile Barred Plymouth Rock eggs were used throughout the experi-

ments being reported. Unless otherwise stated, these were incubated for ten days prior to inoculation. Bacteriologically sterile spleens from calves ill of rinderpest and killed on the second or third day of fever were used as the source of rinderpest virus in starting each series of egg cultures. The eggs were prepared for inoculation by cutting, with a dental drill, a small square window in the shell over the region of the embryo. The air sac was then collapsed by suction through a small hole drilled in the air sac end of the egg. This created an artificial air sac under the opening in the shell and exposed a large area of chorioallantoic membrane for inoculation. Two-tenths cc. of a 10 per cent suspension of virus-infected spleen in broth was dropped on the chorioallantoic membrane of each egg in the first passage series. The window in the shell was then covered with scotch tape and the small opening at the air sac end with heated sealing wax.

Inoculated eggs were incubated for three days, unless otherwise stated, before opening for examination and serial passage. The shell about the opening was cut away with scissors, exposing the surface of the inoculated portion of the chorioallantoic membrane. The portion of this membrane thus exposed was removed aseptically, as was also the underlying amniotic membrane, and placed in a sterile pyrex glass grinder (Ten Broeck). Then about 3 cc. of the combined allantoic and amniotic fluids were removed by pipette and also placed in the grinder. Material from three eggs was collected in this way at each passage and finely ground. Two-tenths cc. of this membrane suspension in egg fluid was then used to inoculate the chorioallantoic membrane of each egg of the subsequent serial passage. A larger amount was planted on blood agar to test for bacteriologic sterility.

RESULTS

As mentioned earlier, three separate sets of egg cultures were started and carried in series during the time that we were experiencing difficulties in maintaining constant temperatures in our incubators.

Fourth serial passage membranes of the first set of cultures were tested for the presence of rinderpest virus by inoculation of a calf. This animal came down with rinderpest after an incubation period of three days. The second test of this set of cultures was at the seventh serial passage and this membrane suspension induced a two-day fever in the calf to which it was administered after an incubation period of five days. The animal made a prompt, complete, and uneventful recovery. The third test of the first set of cultures was at the thirteenth serial passage, and the inoculated calf remained normal, indicating that we had lost the virus some time after the fourth serial egg passage.

The first test for rinderpest virus in the second set of egg cultures was made at the fourth serial passage. The calf inoculated with a membrane suspension of this passage developed a two-day febrile reaction and then made a prompt and uneventful recovery. It thus duplicated the result obtained with seventh passage cultures of the first set of eggs. The question of whether or not this mild, two-day ailment was rinderpest or related to it has not yet been completely clarified. What evidence there is at hand, however, indicates that the agent responsible for the transient febrile reaction was indeed attenuated rinderpest virus. The administration of membrane suspensions from the fifth and sixth serial passages of the second set of egg cultures failed to induce any evidence of infection in calves, and the cultures were discontinued.

The first and only test of the third set of egg cultures was made at the fifth serial passage. The calf inoculated with a membrane suspension of this passage came down with characteristic rinderpest. By this time, our small bacteriologic incubators had arrived and we had determined that they could be accurately adjusted to maintain constant temperatures. There was also the suggestion by now that our difficulties in maintaining rinderpest virus through prolonged serial passage in eggs might be in some way related to the extremely low incubator temperatures to which it was sub-

jected in our fluctuating incubator. Consequently, the third set of egg cultures was discontinued after the test of the fifth passage material, and new egg cultures were initiated from infected calf spleen to be carried in incubators maintaining constant temperatures.

Because we had no idea as to what incubation temperature might be most favorable to the development of rinderpest virus in eggs, except the suggestion that low temperature might be unfavorable, it was decided to start three sets of cultures and carry them at three different incubator temperatures. The three temperatures arbitrarily selected were 35 C., 37 C., and 39 C. The cultures were carried for four serial passages before testing in calves. By the fourth passage, a dilution of the virus used in initiating the cultures which was well beyond the known minimal infective dose had been reached. All 3 calves inoculated with suspensions of fourth passage membranes came down with rinderpest. The calves receiving the virus that had been carried at 37 C. and 39 C. died, while the 1 receiving the 35 C. culture eventually recovered. It is not yet known whether any significance can be attached to the failure of virus cultivated in eggs at 35 C. to kill. However, on the chance that it might be of significance, we have quite arbitrarily chosen a higher temperature for use in all of our subsequent egg work. An incubation temperature of 37.5 to 38 C. has been empirically selected and, unless otherwise stated, this temperature has been used in all of the subsequent experiments to be outlined in this report.

TITRATION OF RINDERPEST VIRUS IN THE CHORIOALLANTOIC AND AMNIOTIC MEMBRANES OF INFECTED EGGS

Infected membranes in which the virus content was to be titrated were harvested in the usual way, weighed in tared Petri dishes and suspended to make either 10 per cent or 20 per cent suspensions in mixed allantoic and amniotic egg fluids. As will be pointed out in more detail later, these fluids have so far been found free of virus and, hence, their use in the initial dilution has no quantitative bearing on the virus titers found. All subsequent dilutions have been made in beef infusion broth, and no effort has been made to titrate the virus closer than by dilution intervals of 10. In testing for the presence of virus, 1-cc. amounts of the dilution have been administered subcutaneously to calves.

The egg-culture virus used in both titrations to be outlined had as its origin the egg-passage series that had been carried at 37 C. and 39 C., described earlier. When both of these were found to contain active virus at the fourth passage, membrane suspensions of their third serial passages that had been stored in a frozen state were thawed, pooled, and used to infect a second set of fourth passage eggs. The egg passages were then continued serially in an incubator whose temperature was adjusted to 37.5-38 C.

The first titration was conducted using infected membranes from the fourth serial egg passage. The results are shown in table 1.

TABLE 1—Titration of Rinderpest Virus in Mixed Chorioallantoic and Amniotic Membranes of Fourth Serial Egg-Passage Cultures

Calf No.	Dilution of Membranes*	Results
29	1-10	Rinderpest
30	1-100	Rinderpest
38	1-1,000	Rinderpest
37	1-10,000	Negative
36	1-100,000	Rinderpest**

*1 cc. of dilution administered subcutaneously as test dose.
**Eight-day incubation period.

The second titration was conducted with infected membranes from the ninth serial egg passage. Instead of using the customary 10-day incubated eggs, 8-day eggs were inoculated at the ninth passage and these were then incubated for five days at 37.5-38 C. prior to harvesting their membranes for titration. Instead of utilizing only those portions of the membranes that could be removed through the opening in the egg shell, the entire egg contents were poured into Petri dishes and the complete chorioallantoic and amniotic membranes stripped away. The results of the titration of these membranes are shown in table 2.

TABLE 2—Titration of Rinderpest Virus in Mixed Chorioallantoic and Amniotic Membranes of Ninth Serial Egg-Passage Cultures

Calf No.	Dilution of Membranes*	Results
37-B	1-50	Rinderpest
35	1-500	Rinderpest
45	1-5,000	Rinderpest
44	1-50,000	Negative
43	1-500,000	Negative

*1 cc. of dilution administered subcutaneously as test dose.

Although the two titration experiments recorded are not strictly comparable because of the differences in the time that the virus was allowed to grow, they do indicate that fairly high titers of rinderpest virus are achieved by cultivation in eggs. In the second experiment, for instance, the weight of the membranes harvested for the titration averaged 2 Gm. per egg, indicating that each egg yielded at least 10,000 minimal infective calf doses of rinderpest virus. This compares quite well with the yield of virus obtainable from the best bovine tissues. It is conceivable that the optimum yield of virus from eggs may be obtained in cultures incubated for a period intermediate between three and five days. This possibility is being investigated at the present time. It is of interest that, when the egg cultures are transferred at only two-day intervals, rinderpest virus is lost by the fourth serial passage. This observation indicated that transfer at less than three-day intervals should not be practiced and suggests the likelihood that longer incubation up to certain, as yet undetermined, limits may result in optimum yields of virus. Transfer at three-day intervals seems to constitute a satisfactory procedure for ordinary purposes of serial passage in eggs and at the present time the virus has been successfully carried in this way through 11 serial transfers.

An explanation for the irregularity of end point in the first titration experiments is not apparent. Calf 37, which received the 1:10,000 dilution and failed to sicken in spite of the fact that calf 36 receiving the next tenfold higher dilution did, was subsequently tested for susceptibility and found to be susceptible. Calf 36 was atypical in that it had the unusually long incubation period of eight days.

DISTRIBUTION OF VIRUS IN THE EGG

In eggs, inoculated when they have embryonated for ten days and transferred at three-day intervals, virus has so far proved to be present only in the membranes. Since we have made it a routine practice to harvest both the chorioallantoic and amniotic membranes at each passage and test these 2 sets of membranes pooled, it is not yet known whether both or only one of the membranes is infected. It seems most certain that, if only one of the membranes is infected, it is the chorioallantoic because the inoculum is placed directly on this membrane at each passage. Lesions that could be considered pathognomonic of rinderpest virus infection have not been observed on either the chorioallantoic or amniotic membranes, although the former is quite regularly thickened, grey, and variably edematous. The specificity of these changes, however, seems doubtful. The virus in the number of passages so far carried out has not appeared to injure the embryos of infected eggs.

Two tests, by calf inoculation, for the presence of virus in mixed allantoic and amniotic fluids have been negative, as have also 3 tests for virus in embryos of eggs whose membranes have been positive. Tests for the presence of virus in egg fluids and in embryos have been made in passages as advanced as the ninth serial transfer in eggs whose membranes had contained virus at every transfer. It is hoped, after prolonged enough passage on the membranes of embryonating eggs, that the virus will eventually become invasive for the embryos. This would increase tremendously the yield of virus, from an individual egg, which could be used in the preparation of vaccine.

There is evidence, furnished by a single experiment, that the use of eggs containing younger embryos and incubated for longer than the usual three days after inoculation may hasten the adaptation of rinderpest virus to the embryo. In this particular experiment, eggs that had been embryonated

for eight days were inoculated in the usual way on the chorioallantoic membranes. They were then incubated for five days before harvesting membranes and embryos to test for virus. Virus was demonstrated, by the inoculation of calves, both in a suspension of embryos and of the membranes. If such findings prove reproducible, it seems likely that serial passage of the virus at longer intervals may hasten its adaptation to the embryo. The series of cultures are being continued to test the possibility.

THE EFFECT OF TEMPERATURE OF INCUBATION ON THE DEVELOPMENT OF RINDERPEST VIRUS IN EGGS

At the beginning of this report, it was pointed out that our early attempts to cultivate rinderpest virus in embryonating eggs were handicapped by power failures and by an incubator whose temperature fluctuated between 32 C. and 38 C. We suspected that our inability to propagate the virus through any prolonged series of egg passages at that time might have resulted from the inability of the virus to multiply at low temperatures. To test the correctness of this opinion, two series of egg cultures were initiated from the same infected spleen suspension. One series was incubated at 32 C. and the other at 38 C. Each series was transferred to a new set of 10-day embryonated eggs every three days in the usual fashion. The embryos in both sets of eggs survived and developed, though those in the eggs incubated at 38 C. developed most rapidly. After 5 serial transfers, the membranes of each set of eggs were tested for the presence of rinderpest virus by the inoculation of calves. The membrane suspension from the fifth passage eggs that had been incubated at 38 C. induced characteristic rinderpest in the calf to which it was administered. The fifth passage membrane suspension from the eggs incubated at 32 C., however, failed to induce any evidence of infection in the calf to which it was given. These findings indicate that incubation at low temperatures is unfavorable to the multiplication of rinderpest virus in embryonating eggs. They suggest that our early failures to maintain the virus through more than a few serial passages may have been due to this cause.

SUMMARY

1) Rinderpest virus has been cultivated for 11 serial passages on the chorioallantoic membranes of embryonating hens' eggs.

2) The titer of virus grown in eggs compares favorably with the titer of that in bovine tissues ordinarily used in the preparation of anti-rinderpest vaccines.

3) In eggs inoculated when embryonated for ten days, the virus after three days' growth is present in the membranes but not in the fluids or embryos.

In a single experiment, 8-day eggs that had been incubated for five days after inoculation contained virus not only in the membranes but in the embryos as well. There is thus every likelihood that rinderpest virus can eventually be adapted to regular multiplication in the embryos, thereby increasing the potential quantitative yield of virus per egg.

4) The most favorable incubation temperature for the cultivation of rinderpest virus in eggs seems to be in the neighborhood of 37 C.-39 C. At 35 C., there is evidence that egg-cultivated virus becomes attenuated, while at 32 C. the virus cannot be maintained in serial egg passages.

Rinderpest
II. Certain Immunity Reactions

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IMMUNITY of cattle recovered from either experimentally produced or naturally occurring rinderpest to infective doses of the causative virus is well established. Furthermore, serums from such animals are capable of protecting others against infection. Studies of the manifestations of this immunity were indicated in the search for a method of diagnosis based on *in vitro* serologic tests. It has been shown for many virus diseases that suspensions containing the active agents, when mixed with appropriate immune serums, are neutralized and do not produce disease when inoculated into susceptible animals. The complement fixation test also has been used successfully. Both methods offered possibilities and the results of their investigations are presented herewith.

THE NEUTRALIZATION TEST

A portion of calf spleen containing virus was weighed and a 10 per cent suspension in physiologic salt solution made by trituration with sand. This suspension was centrifuged for five minutes at 1,200 r.p.m. Tenfold dilutions of the supernatant were prepared and placed at a temperature of 34 F. for 2 hours. Calves were inoculated subcutaneously with 1 cc. of each dilution to determine the number of minimal infective doses (m.i.d.) contained in a gram of spleen.

Based on these titrations, similar suspensions containing 1,000 (10^{-3} dilution) and 10,000 10^{-4} dilution) m.i.d.'s in each cubic centimeter were prepared and mixed with an equal amount of the serum to be tested. Following an incubation period of two hours at 34 F., 2 cc. of each mixture were given to a calf subcutaneously. Absence of clinical signs in the test animal, and its susceptibility to a subsequent injection of active virus, determined neutralization.

Serums were taken from 3 calves that had recovered from the disease. One animal (3) had been infected by contact, another (215) had been given an injection of bovine spleen containing virus, while the third (41) had been infected with egg-propagated virus. Serum was drawn from calf 3, fifty-two days after the onset of fever; from calf 215, twenty-eight days after, and from calf 41, thirty-two days afterwards. Calf 3 had received an intermediate injection of 10 cc. of a 10 per cent spleen suspension containing active virus as an attempted hyperimmunizing injection. A portion of the serum from calf 3 was further modified by drying from the frozen state and reconstituting with distilled water before testing. Control serums were taken from 2 calves (22 and 12), shown to be nonimmune by later injections of active virus. The results are shown in table 1.

It may be seen in table 1 that 1,000 infective doses of rinderpest virus were neutralized consistently by convalescent serums. The injection of a large amount of virus during convalescence probably increased the neutralizing titer of the serum from calf 3. Serums dried from the frozen state when reconstituted showed some loss in the capacity to neutralize. Normal serum does not alter the course of the disease when 1,000 m.i.d.'s are given to calves.

THE COMPLEMENT FIXATION TEST

Calf spleens containing virus were ground with sterile sand and made up to a 10 per cent suspension. This suspension was centrifuged for thirty minutes at 2,500 r.p.m.

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and the sediment discarded. The supernatant, diluted 1:5, constituted the antigen used in early tests, and it was not anticomplementary. This antigen, prior to dilution, was again centrifuged at 15,000 r.p.m. for one hour, a procedure known to sediment all demonstrable active virus in spleen. The supernatant was poured off and the sediment resuspended in salt solution to the original volume. This, diluted 1:5, was the antigen used in most tests and it also was not anticomplementary when two and a half times the usual amount employed was added. By this procedure, a slightly turbid antigen was produced that was free of the hemoglobin present in the material centrifuged at low speed. Thus, easier although completely comparable readings were obtained.

Nonimmune serums were collected from normal susceptible calves. Calves recovered from typical disease and resistant to further infection with active virus furnished the immune serums. All serums were inactivated by heating at 56 C. for thirty minutes.

Each tube contained antigen, serum, complement (2 units in 0.5 cc.), and salt solution to bring the total volume to 2 cc. A constant amount (0.5 cc.) of antigen and decreasing amounts of serums were used. The tubes were incubated overnight in the refrigerator. One cubic centimeter of a mixture of equal parts of 2 per cent washed sheep red blood cells and hemolytic amboceptor diluted to contain 2 units in each 0.5 cc. was added and readings were made after thirty minutes incubation at 37 C. Adequate controls were included. The results are given in table 2.

TABLE 1—Neutralization Tests with Virus of Rinderpest

Test Calf No.	Number of m.i.d.'s of virus in test inoculum	Serum tested		Results	
		From Calf No.	Status	Injection of serum-virus	Later inoculation of virus
35	10,000	3	Immune	No illness	Rinderpest
81	10,000	3	Dried immune	Rinderpest	.....
99	1,000	3	Dried immune	No illness	Rinderpest
261	1,000	3	Dried immune	No illness	Rinderpest
89	10,000	41	Immune	Rinderpest	.....
101	1,000	41	Immune	No illness	Rinderpest
262	1,000	215	Immune	No illness	Rinderpest
34	10,000	22	Normal	Rinderpest	.....
82	10,000	22	Normal	Rinderpest	.....
91	1,000	12	Normal	Rinderpest	.....
102	1,000	12	Normal	Rinderpest	.....

TABLE 2—Complement Fixing by Serums of Convalescent Calves with Rinderpest Virus Antigen from Calf Spleens

Antigen	Serums tested			
	Nonimmune		Immune	
	No. 22	No. 12	No. 3	No. 41
Infected Spleen No. 29	X>1/2	Not tested	1/8	Not tested
Infected Spleen No. 38	>1/2	Not tested	1/8	Not tested
Infected Spleen No. 62	>1/2	Not tested	1/8	Not tested
Infected Spleen No. 91	>1/2	>1/2	1/16	1/16
Normal Spleen No. 1	>1/2	>1/2	1/2	1/2

X>1/2 = No fixation of complement.

Complement fixing antibodies in equivalent amounts were demonstrated in the serums of 2 calves recovered from rinderpest. No significant difference in the amounts of complement fixed were found with antigens from 4 calves. Normal spleen or nonimmune serums did not fix complement.

DISCUSSION

The evidence presented shows that previous infection of calves by rinderpest virus can be detected by the neutralization and complement fixation tests. Of the two, the neutralization test is to be preferred for its accuracy and simplicity. Complement fixing antibodies are of such low titer as to be undependable. For diagnosis, therefore, the method of choice would be the neutralization test and the following procedure is suggested.

During an outbreak of an infectious disease, in which rinderpest is a possibility, an animal showing acute signs should be killed and a 10 per cent suspension of the spleen in physiologic salt solution prepared by maceration. After sedimentation of large particles, either by standing or centrifugation, a 1:10 and 1:100 dilution of the supernatant should be made with physiologic salt solution. One cubic centimeter of these dilutions should represent, respectively, approximately 1,000 and 100 m.i.d.'s of rinderpest virus. When mixed with an equal amount of immune serum, the virus in these dilutions should be neutralized, as indicated by the failure of the mixtures when administered subcutaneously to calves to cause illness. A part of the same suspensions mixed with normal serum and similarly tested should produce typical signs of infection in normal calves.

SUMMARY

Serums from calves recovered from rinderpest have the power of fixing complement when mixed with suspensions of spleen containing the virus. However, the titers of such serums are too low to yield dependable results. Rinderpest virus can be neutralized *in vitro* by serums of calves recovered from rinderpest. Such serums in 1-cc. amounts will neutralize at least 1,000 minimal infective doses. On the basis of the neutralization test, a method of diagnosis is discussed.

Rinderpest

III. Immunization Experiments with Inactivated Bovine Tissue Vaccines

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THE RINDERPEST vaccine that we have prepared is a modification of the one devised by Kelser, Youngberg, and Topacio[1] and used by them with highly satisfactory results in controlling rinderpest in the Philippines. In brief, the Kelser vaccine consisted of a 50 per cent suspension in physiologic salt solution of finely ground, infected spleen, lymph nodes, and liver inactivated with 0.75 per cent of chloroform. Three doses of 15 to 20 cc. of this vaccine administered subcutaneously at weekly intervals solidly immunized cattle or carabaos against rinderpest. A single injection of 25 cc. of the vaccine also gave immunity. Rodier,[2] modifying the Kelser vaccine only by omitting infected liver in its preparation, produced a vaccine of such potency that a single injection of 10 or 20 cc. subcutaneously immunized cattle or carabaos, respectively.

At the time that we began our attempts at the production of a rinderpest vaccine satisfactory for possible use in the probably highly susceptible cattle of nonenzoötic areas such as Canada and the United States, we had the advantage of valuable personal advice from General Kelser. We also had been informed that investigators working with the disease elsewhere had found lungs to be extremely rich in virus and to be highly satisfactory for inclusion as one of the tissue constituents of rinderpest vaccine.

In order to obtain information other than that available in the literature as to the tissues richest in virus and, therefore, most probably satisfactory for inclusion in our vaccine, various organs and tissues were titrated in experimental calves for their virus content. Animals that had been febrile of rinderpest for two or three days were chosen as a source of tissues for titration. It was found that lymph nodes, spleen, and lungs contained sufficient virus so that a 1:10,000 suspension of these tissues in physiologic salt solution was infective for experimental calves and that the incubation periods for such infections were not prolonged beyond the customary three days. Kidney and liver suspensions failed to infect at this dilution, while defibrinated blood, though infective, brought animals down only after a lengthened incubation period. Because of these findings, it was decided to include only lungs, spleen, and body lymph nodes in our proposed vaccine. The mesenteric lymph nodes were omitted because of the possibility, pointed out by earlier workers, that these nodes might contain pathogenic spore-bearing organisms which would not be destroyed by the concentration of chloroform we contemplated including in our vaccine. The omission of the mesenteric lymph nodes made a negligible difference in the total amount of vaccine that could be prepared from the utilizable tissues of an animal.

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The monthly reports occasionally referred to in this publication are official reports on the progress of the project, rendered monthly to the U. S.-Canadian Commission and filed in the Office of the Chief of Chemical Warfare. Interim Reports are special reports on important scientific accomplishments and developments in the project and rendered at such times as developments and circumstances warranted. Most of these Interim Reports constitute the basis of the present publication.

PREPARATION OF VACCINE

Healthy calves ranging in age from 4 to 12 months were used in the production of vaccine. They were inoculated subcutaneously either with egg culture rinderpest virus[3] or with bovine splenic virus. Such animals became febrile on either the second or third day after inoculation and developed the characteristic signs of rinderpest. They were killed by stunning and bleeding on the second or third day of fever. In order to prevent the introduction of blood or extraneous material into the respiratory tract, the tracheas were clamped shut following stunning but before bleeding to death. A sterile container was used to receive the organs to be saved for vaccine production. The body lymph nodes were removed aseptically first. Then the abdomen was opened and the spleen removed aseptically. After this, the thorax was opened and the lungs removed aseptically, lobe by lobe, using long sponge forceps to grasp the lobes as they were cut free of the larger bronchi at the hilum. After removal of the organs that were to be saved for vaccine production, the remainder of the autopsy was conducted in the routine manner. Aërobic and anaërobic cultures were made of the spleens in all cases and similar cultures were prepared from any pneumonic areas of the lungs that seemed of such an age as to antedate the rinderpest infection. Tissues yielding pathogenic organisms were discarded.

Immediately following the autopsy, the tissues that had been saved for vaccine preparation were taken to the production room where they were finely minced in a sterilized Hobart (model 4212) meat chopper, passing them once through a "stay sharp chopper plate" with 3/16-in. holes. This finely ground material was collected in tared, sterile, enamel utility jars of 6- to 7-quart capacity. Usually, the ground tissues from 2 calves were placed in a single jar. The filled jars were next weighed to ascertain the amount of material they contained and were then placed in the freezing room for storage at —5 F. until used in the preparation of vaccine. When stored frozen at this temperature, the virus can be maintained without loss of titer or virulence for long periods of time, at least seven months.

When it was desired to prepare vaccine, utility jars containing the frozen, ground, infective tissues were removed from the freezing room and placed in an incubator to thaw. Since the colloid mill we use in the preparation of vaccine has a maximum working capacity of 15 liters, only tissue sufficient to make approximately this amount of vaccine was removed from the freezer at one time. After thawing, the ground tissue, together with about an equal amount of sterile 0.85 per cent salt solution, was placed in the hopper of the colloid mill. The mill we have used is an Eppenbach (model Q V 6-1). The grinding knives of the mill were set at space 70 in starting and run for five minutes at this setting. The adjustment was then closed to 60, and then later to 50, and run for three minutes at each of these settings. By this time, the coarser particles of lung which tend to float in the hopper at the beginning had been sufficiently homogenized so that they no longer stayed on the surface of the fluid in the hopper. At this stage, the remainder of the sterile 0.85 per cent salt solution required to make a 20 per cent by weight tissue suspension was added. The mill was then run for periods of approximately two minutes each at settings 40, 36, 32, 28, 24, 20, 18, 16, 14, and 12. At the end of this series of runs, the tissue suspension was evenly homogenous and of such fineness that it could be readily drawn through a 19-gauge Luer needle. The finished suspension was drawn off into sterile bottles with the mill running at setting 12. The inactivating agent (chloroform or formalin) was sometimes added to the suspension immediately after it had been bottled. The usual procedure has been, however, to add the required amount of either chloroform or formalin to the suspension while it was still in the mill and then to run the mill an additional minute to permit of thorough mixing. When formalin was used in inactivating the virus for vaccine, it was added to make a 1:1,000 concentration. Chloro-

form, which has been most largely used by us, has been added in the concentration recommended by Kelser, namely 0.75 per cent.

The product of a single run of the mill has been designated as a batch and consists of approximately 15 liters of vaccine. It is stored in 9-liter Pyrex serum bottles in the refrigerator (at ±2 C.) and is shaken daily for the first four days after preparation. Experience has shown that vaccines prepared using chloroform as the inactivating agent become bacteriologically sterile before the sixth day of refrigeration, even though they may have been lightly contaminated when first prepared. Formalin-inactivated vaccines require about twice this length of time to sterilize. Each batch of vaccine is cultured aërobically on horse-blood agar and anaërobically in Brewer's thioglycholate medium. All batches of chloroform-inactivated vaccine that we have prepared to date have been bacteriologically sterile when cultured six days after preparation. The formalin-inactivated vaccines have also eventually become bacteriologically sterile. As will be pointed out later, they are of less immediate practical interest because they may be slightly inferior to the chloroform vaccines in immunizing potency.

When several batches of chloroformized vaccine have been prepared and found to be bacteriologically sterile, they are pooled in sterile 50-liter bottles and thoroughly mixed to make what we have designated as a "lot." Each lot is comprised of either 3 or 4 batches of vaccine. After mixing, the contents of the lot is distributed in 500-cc. amber serum bottles, capped with vaccine type rubber stoppers, labeled and stored in the refrigerator (at ±2 C.).

A series of control tests are run on the contents of the last bottle of each lot of vaccine as follows: (a) The vaccine is again checked for bacteriologic sterility by culture on blood agar and in Brewer's medium; (b) 1 cc. is administered subcutaneously to a guinea pig and 0.2 cc. intraperitoneally to each of 6 white mice; (c) a calf is vaccinated with 10 cc. of the vaccine. To be acceptable, a lot of vaccine must prove to be bacteriologically sterile by the culture methods used, it must be innocuous to the guinea pigs and white mice during one month's observation, and 10 cc. must immunize a calf against a challenge dose of 100 to 1,000 minimal infective doses of rinderpest virus administered two weeks after vaccination.

Proper sterilization of the machines used in producing the vaccine as outlined is of importance not only from the standpoint of attaining a sterile end-product but also to minimize the danger of the dissemination of rinderpest virus by indirect means. The working parts of the Hobart meat chopper are cleaned in scalding water after use and thus rendered non-dangerous as far as virus dissemination is concerned. Prior to use, the parts are assembled, wrapped and autoclaved. The Eppenbach mill is not quite so easy to sterilize. After use, it is filled with 5 per cent formalin for one and a half hours and then rinsed and washed thoroughly with scalding water. It is then disassembled, its cutting parts thoroughly cleaned mechanically in scalding water, and covered with a thin coating of petroleum jelly to prevent rusting. Prior to use, it is assembled and filled with 5 per cent formaldehyde for one and a half hours. Then it is thoroughly washed out, while running, with two changes of scalding water. After this, the machine is filled with cold boiled water. This is drained out with the machine running after it has cooled the metallic working parts of the mill. The hopper is covered with a sterile towel during the milling process.

TEST OF THE IMMUNIZING CAPACITY OF INACTIVATED BOVINE TISSUE RINDERPEST VACCINES

The first batch of vaccine that we prepared was made using the infected spleen, lungs, and lymph nodes from a single animal, calf 38. The spleen from this particular calf proved infective in a dilution of 1:100,000 instead of attaining only the usual titer of 1:10,000. We are not aware of the reason for this unusually high virus titer, but efforts to duplicate it in other

calves, imitating as nearly as possible the procedures used in the case of calf 38, have not been regularly successful. The calf had been infected with fourth egg-passage rinderpest virus.

The results of tests of the vaccines prepared from the tissues of calf 38 are outlined in table 1.

deavored to learn whether these rather heroic doses could be reduced without jeopardizing the immunity attained. It was found, successively, that single doses of vaccine in either 25-cc. or 12.5-cc. amounts resulted in solid immunity regardless of whether the inactivating agent employed was chloroform or formalin. When the dose

TABLE 1—Immunization against Rinderpest with Formalin- and Chloroform-Inactivated Tissue Vaccines from a Single Animal

Calf No.	Vaccine inactivated with	Immunization		Challenge infection with 100-1,000 m.l d.'s rinderpest virus
		Amt. vaccine per injection (x)	No. subcutaneous injections	
55	Formalin 1-1,000	25 cc.	3	Negative (immune)
56	Formalin 1-1,000	25 cc.	3	Negative (immune)
107	Formalin 1-1,000	25 cc.	1	Negative (immune)
136	Formalin 1-1,000	12.5 cc.	1	Negative (immune)
137	Formalin 1-1,000	5 cc.	1	Positive (mild illness—recovered)
205	Formalin 1-1,000	5 cc.	1	Negative (immune)
58	Chloroform 0.75%	25 cc.	3	Negative (immune)
64	Chloroform 0.75%	25 cc.	3	Negative (immune)
65	Chloroform 0.75%	25 cc.	3	Negative (immune)
106	Chloroform 0.75%	25 cc.	1	Negative (immune)
134	Chloroform 0.75%	12.5 cc.	1	Negative (immune)
135	Chloroform 0.75%	5 cc.	1	Negative (immune)
195	Chloroform 0.75%	3 cc.	1	Negative (immune)
194	Chloroform 0.75%	1 cc.	1	Positive (2-day mild illness—recovered)
98	Control (not vaccinated)			Positive (died 8th day)
103	Control (not vaccinated)			Positive (died 8th day)
142	Control (not vaccinated)			Positive (died 9th day)
215	Control (not vaccinated)			Positive (typically ill but eventually recovered)
232	Control (not vaccinated)			Positive (died 8th day)

x = 20% tissue suspension in 0.85% sodium chloride solution.

This is a composite table, since all of the tests recorded were not done at the same time. In the earlier tests, we followed Kelser's general procedure of giving 3 rather large doses of vaccine at weekly intervals. All 5 of the animals that received 3, 25-cc. injections of vaccine, either formalin- or chloroform-inactivated, proved immune to a challenge dose that was fatal for an unvaccinated calf. In later tests, we en-

of vaccine was reduced to 5 cc., however, only 1 of the 2 calves receiving the formalin-inactivated vaccine proved solidly immune on challenge with active virus. The other underwent a mild rinderpest from which it recovered. The chloroform-inactivated vaccine, on the other hand, solidly protected in 5-cc. and 3-cc. amounts, and a calf that received only 1 cc. was partially protected. This last animal underwent a

mild, two-day, febrile illness from which it promptly recovered.

While no exhaustive attempts have been made to further evaluate the relative merits of the chloroform-inactivated vaccine as compared with the formalin - inactivated one, the evidence that we have indicates a slight margin of superiority for the former. This, considered with the more rapid sterilization of tissue suspensions by chloroform, has influenced us in favor of the Kelser vaccine for possible use in the present emergency.

In table 2 are shown the results of a series of tests of various lots and two individual batches of chloroform-inactivated vaccine for their capacity to immunize. As was pointed out earlier, a lot is comprised of a pool of several batches of vaccine and contains material from either 5, 6, or 7 calves, depending upon the tissue weights of the animals included. For various reasons, it is impractical to titrate the tissues of each calf going into a lot for its virus content. However, we do know from experience with infected tissues that have been

TABLE 2—Immunization against Rinderpest with Various Lots of Chloroform-Inactivated Tissue Vaccines Each Containing Pooled Tissues from Several Animals

Calf No.	Designation of vaccine	Immunization		Challenge infection with 100-1,000 m.i.d.'s rinderpest virus
		Amount of vaccine per injection (x)	Number of subcutaneous injections	
111	Lot 1	25 cc.	3	Negative (immune)
225	Lot 1	10 cc.	1	Negative (immune)
179	Lot 1	5 cc.	1	Positive (2-day mild illness—recovered)
118	Lot 2	25 cc.	1	Negative (immune)
226	Lot 2	10 cc.	1	Negative (immune)
180	Lot 2	5 cc.	1	Positive (2-day mild illness—recovered)
227	Lot 3	10 cc.	1	Positive (killed 7th day of illness)
181	Lot 3	5 cc.	1	Positive (5-day illness—recovered)
267	Lot 4	10 cc.	1	Negative (immune)
182	Lot 4	5 cc.	1	Temperature elevation on 5th day—no illness
229	Lot 5	10 cc.	1	Negative (immune)
183	Lot 5	5 cc.	1	Positive (1-day mild illness—recovered)
252	Lot 6	10 cc.	1	Negative (immune)
230	Lot 6	10 cc.	1	Negative (immune)
184	Lot 6	5 cc.	1	Positive (1-day mild illness—recovered)
268	Batch 21	10 cc.	1	Negative (immune)
269	Batch 22	10 cc.	1	Negative (immune)
198		Control (not vaccinated)		Positive (typically ill but eventually recovered)
232		Control (not vaccinated)		Positive (died 8th day)
245		Control (not vaccinated)		Positive (died 9th day)
260		Control (not vaccinated)		Positive (died 7th day)
279		Control (not vaccinated)		Positive (died 9th day)

x = 20% tissue suspension in 0.85% sodium chloride solution.

titrated, that a dilution of 1:10,000 more frequently represents the m.i.d. than does the dilution of 1:100,000 found in the case of calf 38 which supplied tissues for the vaccine recorded in table 1. It is a reasonable assumption, therefore, that most of the lots of vaccine contained less titratable virus than did the batch with which the original work recorded in table 1 was carried out. It is believed that this difference may account for the larger doses required to immunize in the case of the lots. As is shown in table 2, a single 5-cc. dose of no lot of vaccine gave complete protection against the challenge injection of rinderpest virus. Five of the 6 lots, tested in 5-cc. dosage, however, gave sufficient protection so that the challenge dose of virus induced only a mild, transient, one- or two-day illness manifested mainly by a febrile reaction. Lot 3 conferred little immunity in 5-cc. dosage and was also ineffective even when a single 10-cc. dose was employed. Nothing in the case histories or autopsy findings of the calves supplying the tissues used in the preparation of lot 3 gave a clue to explain its ineffectiveness. The lot was, however, discarded as unsatisfactory for use. All of the remaining 5 lots gave complete protection to calves when single injections of 10 cc. were employed.

Sufficient time has not yet elapsed to completely test the keeping qualities of the vaccine. However, a single 3-cc. injection of batch 1, our oldest preparation, completely protected a calf when used after fifteen weeks of refrigerator storage.

That the temperature at which the vaccine is stored is of importance in determining its keeping qualities is indicated by an experiment with lot 6 vaccine. In this experiment, one bottle of the vaccine was incubated at 38 C. for eight days. Then 1 calf was given a single subcutaneous injection of 15 cc. of the incubated lot 6 vaccine, while 10 cc. of the same vaccine kept under the usual refrigeration conditions was given to a second calf. Two weeks later, the animals received their challenge dose of rinderpest virus. The one vaccinated with 10 cc. of refrigerated vaccine remained normal. However, the calf that had received 15 cc. of incubated vaccine developed characteristic rinderpest and died on the thirteenth day.

Practically all of our immunization work has been conducted with young animals ranging in age from 4 to 12 months. In order to obtain some idea as to whether or not older animals could be protected by a dosage of vaccine similar to that found effective in calves, an 8-year-old cow was given a single subcutaneous injection of 10 cc. of lot 6 vaccine. It was challenged two weeks later with virulent rinderpest virus which proved fatal for a control animal. The cow remained normal. So far as this evidence goes, it indicates that the dosages of vaccine required by immature and adult animals are similar.

DISCUSSION

We have prepared a vaccine which, so far as can be told from tests under experimental conditions, is satisfactory for use in protecting cattle against rinderpest. The vaccine, as prepared, is a very slight modification of the one developed and used successfully in the Philippines by Kelser and his coworkers. We have on hand a sufficient amount of finished vaccine to combat initial, scattered outbreaks of the disease and we have, further, in frozen storage a relatively large amount of infected tissue which can, on short notice, be made into vaccine.

If our vaccine is to be employed at all in Canada or the United States, it will be only as an emergency procedure and in the face of an outbreak of rinderpest. Under such a circumstance, there are very obvious disadvantages to the use of a vaccine requiring multiple inoculations to immunize. We have, therefore, directed our efforts towards the production of one which will confer satisfactory immunity after a single administration. So far as we can determine under experimental conditions, a single injection of 10 cc. of the vaccine is adequate to confer protection. It is believed that this dosage would in all probability be adequate for field use providing the vaccine can be transported to places where it is to be used

without diminution in the immunizing capacity it possesses when it leaves here. However, because of the possibility that during transportation the vaccine may be submitted to deleterious conditions and, as an added precaution in the face of an emergency, it would probably be safer to place the recommended field dose at 20 cc. per head subcutaneously. It must be borne in mind that, in case of an epizoötic on the North American Continent, we would have to deal with an outbreak of disease in an entirely susceptible cattle population. The problem of controlling rinderpest by vaccination would, in all likelihood, be quite different and perhaps more difficult than it is in a region where the disease is enzoötic and where some of the animals, at least, possess residual immunity from previous contact with the disease.

Two questions of some importance which should be answered, and which cannot be from the data so far at hand, concern the keeping qualities of the vaccine as prepared and the duration of the immunity in vaccinated animals. While we do not yet know the longest period of time that our vaccine will maintain its immunizing efficacy, we do know that it is still effective in inducing immunity after a period of fifteen weeks' storage in the refrigerator. Kelser[1] has stated that his vaccine maintained its potency for one year and, since ours is such a close replica of his, there seems every assurance that ours will keep as long. We have not yet tested the duration of the immunity conferred by our vaccine though such experiments are at present in progress. Our practice has been to challenge animals two weeks after vaccination and the information we have at present is all based on observations for that period of time. From a practical standpoint in an emergency such as the one we anticipate and on a continent where the disease is not enzoötic, and will probably not be permitted to become enzoötic, the question of the possible duration of immunity is of less vital importance than it is were the vaccine being prepared for use in regions where the disease is enzoötic. Rather, it is more important, for possible use here, to be assured that we have available a vaccine which elicits a prompt immunity response. If the immunity is durable for two or three months, that should be sufficient to handle outbreaks of the type that are visualized as possibilities in the present emergency.

The question of whether or not chloroform-inactivated vaccines are definitely superior to those inactivated with formalin has not been exhaustively enough studied for an intelligent decision at this time. What evidence we have, however, indicates that the chloroform vaccine is at least as good as the formalin one and the greater ease and speed of preparation of the former has decided us in its favor.

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1) The method of preparation of a satisfactory bovine tissue rinderpest vaccine has been outlined.

2) Using high titer virus from a single animal, a vaccine of quite good immunizing potency was prepared.

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6) It is recommended that the dose of rinderpest vaccine for use under field conditions be 20 cc., administered subcutaneously.

References

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Rinderpest

IV. Infection of the Embryos and the Fluids of Developing Hens' Eggs

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IN A PREVIOUS report,[1] the successful cultivation of rinderpest virus on the chorioallantoic membranes of embryonating hens' eggs was described. At that time, the virus had been transferred through 11 serial egg passages. Since then it has been continued under cultivation and has now reached its 50th serial passage on the chorioallantoic membrane. It is still infective for cattle though of considerably diminished pathogenicity.

We had initially attempted to cultivate rinderpest virus in eggs in the hope of obtaining a cheap, dependable, and abundant source of virus for possible vaccine production. While the membranes of infected eggs proved to be relatively rich in virus, they constitute only a small part of the total egg and the yield of wet membranes is seldom more than 2 Gm. per egg. Also, in eggs inoculated on the chorioallantoic membranes, the virus proved to be rather strictly specific for the membranes and apparently did not invade or multiply in the embryos nor accumulate in the egg fluids. Because the yield of virus per egg might be greatly increased if infection of the embryos or fluids could be achieved, efforts were made either to adapt the virus to growth in the embryo or to devise means of inoculation whereby the embryo and perhaps the fluids would become infected. The present report details attempts to alter the distribution of virus in embryonating eggs by varying the route of inoculation.

Received by the Commission on 4 Feb. 1944 for publication which had to be delayed on account of security regulations in effect at that time.

The monthly reports occasionally referred to in this publication are official reports on the progress of the project, rendered monthly to the U. S.-Canadian Commission and filed in the Office of the Chief of Chemical Warfare. Interim Reports are special reports on important scientific accomplishments and developments in the project and rendered at such times as developments and circumstances warranted. Most of these Interim Reports constitute the basis of the present publication.

ATTEMPTS TO INFECT THE EMBRYOS OF EMBRYONATING EGGS BY ADMINISTRATION OF RINDERPEST VIRUS BY VARIOUS ROUTES

1) *By Inoculation of Virus into the Allantoic Sac.*—Third egg-passage virus was introduced into the allantoic sac through the shell and chorioallantoic membranes directly over the embryo. Eggs that had been embryonated for ten days were used and 0.2 cc. of egg-passage virus (a 10 per cent suspension of infected chorioallantoic and amniotic membranes in egg fluids) was introduced into each egg. After three days' incubation at 38 C., chorioallantoic and amniotic membranes and embryos were harvested and washed in three changes of sterile 0.85 per cent salt solution. Separate 10 per cent suspensions in salt solution were then prepared from the pooled membranes and from the embryos. One cubic centimeter amounts of each suspension were inoculated subcutaneously into two susceptible calves to test for the presence of rinderpest virus. Both calves remained normal, indicating that the virus introduced into the allantoic sac had invaded neither the egg membrane nor the embryos.

A similar experiment was conducted with tenth egg-passage virus. In this experiment, the calf receiving the membrane suspension developed characteristic rinderpest

while the calf receiving the embryo suspension remained normal. Here again, virus introduced into the allantoic sac had failed to infect the embryo although it had infected the membranes. Infection of the membranes in this type of experiment was not particularly surprising inasmuch as the needle introducing virus into the allantoic sac had to penetrate the chorioallantoic membrane during the process of inoculation. In a third similar experiment, in which thirteenth egg-passage virus was introduced into the allantoic sacs of 10-day embryonated eggs, neither the membranes nor the embryos were invaded by the virus as indicated by the failure of both membrane and embryo suspensions to infect susceptible calves. Thus, all of three attempts to infect embryos with rinderpest virus by way of the allantoic sac failed and in only one of the three instances were the membranes infected. It was quite apparent that administration of virus by way of the allantoic sac did not constitute a satisfactory means of infecting embryos.

2) *By Inoculation of Virus into the Amniotic Sac.*—Seventeenth egg-passage virus (10 per cent suspension of infected membranes in egg fluids) was introduced directly into the amniotic sac of 10-day embryonated eggs through a window made in the shell over the embryo. After three days' incubation at 38 C., the embryos were harvested, washed in three changes of sterile 0.85 per cent salt solution, suspended in salt solution, and injected into the amniotic sacs of a second group of eggs. After three days' incubation, the embryos of this series of eggs were harvested and tested for the presence of rinderpest virus by inoculation into a susceptible calf. The calf remained normal, indicating that virus introduced into the amniotic sac had not invaded the embryo. This experiment failed to give evidence that intra-amniotic administration of virus constituted a satisfactory method of infecting embryos.

3) *By Inoculation of Virus into the Yolk Sac.*—Very early in our attempts to cultivate rinderpest virus in embryonating eggs, the yolk sac was tried as a possible route of inoculation. Eggs that had been embryonated for five days were used and 0.5-cc. amounts of the inoculum were injected into the yolk sac through a small hole drilled in the shell at the air sac end. The yolk sacs were harvested after three days' incubation, washed, and suspended in 0.85 per cent salt solution either for further serial passage in eggs or for testing for the presence of virus by inoculation into susceptible calves. In the first series of yolk sac-inoculated eggs, virus was present and infective for a calf at the fourth serial passage. Since, at about this time, we discovered that rinderpest virus grew with great facility when inoculated on the chorioallantoic membranes of embryonating eggs, the question arose as to whether or not, in the yolk sac-inoculated eggs, chorioallantoic membranes had been rigidly excluded from the inoculum at each passage. As a consequence, a second series of yolk sac-inoculated eggs was initiated, taking special care this time that only yolk sacs, and no other egg membranes, were included in the inoculum at each serial passage. Under these conditions, no virus could be demonstrated, by the inoculation of a susceptible calf, in the third serial yolk sac passage. Four separate sets of yolk sac-inoculated eggs were started subsequently and carried for four serial passages. In none of these cultures could rinderpest virus be demonstrated, by calf inoculation, in fourth passage yolk sac suspensions. It was concluded after these 5 negative experiments that, in the original positive one, egg membranes other than the yolk sac must have been included in the inoculum at each passage and that this accounted for the apparent cultivation of rinderpest virus in the original yolk sac experiment. It was further concluded that the yolk sac route of inoculation did not constitute a satisfactory means for the propagation of rinderpest virus in eggs and the method was abandoned temporarily.

Later on, after the failure of intra-allantoically or intra-amniotically administered virus to infect embryos, the yolk sac route of inoculation was again studied. It seemed possible that, even though virus apparently did not multiply in the yolk sac when placed

there, it still might infect other portions of the egg either by virtue of its direct invasiveness or by being absorbed from the yolk sac. It was decided to use eggs that had been embryonated for seven days instead of the 5-day eggs used previously and to permit the eggs to embryonate four days after inoculation before harvesting. As will be indicated later, this was a fortunate variation of our original procedure. The yolk sacs were inoculated as before through a hole drilled in the air sac end of the egg, and 0.5 cc. of inoculum was injected into each yolk sac through a 1-in., 21-gauge Luer needle introduced its full length. Also instead of starting our cultures, as we had formerly, with infected bovine spleen, we tried initiating them now with virus that had been under cultivation on the chorioallantoic membranes of eggs for a number of passages. They were incubated at 38 C.

A group of 7-day embryonated eggs were inoculated into the yolk sac, as described previously, with eighteenth egg-passage rinderpest virus (10 per cent suspension of infected membranes in egg fluids). After four days' further incubation, the embryos were harvested and a suspension of the decapitated embryos tested for the presence of virus by calf inoculation. Virus was readily demonstrable. The series was continued by using the embryo suspension to inoculate a second group of 7-day embryonated eggs *via* the yolk sac. The embryos of the second yolk sac-passage proved infective for a calf also. No difficulty was encountered in continuing the passage series in this way, inoculating each subsequent group of eggs into the yolk sacs with embryo suspensions prepared from the preceding passage, or with allantoic and amniotic fluids which, we later found, contained as much virus as did the embryos. Several series of cultures, all of them started with virus that had undergone a number of preliminary passages on the chorioallantoic membrane, have been carried successfully in eggs inoculated by way of the yolk sac as outlined above. No difficulty has been encountered in maintaining any of these. In the most advanced of our yolk sac-transmitted cultures, the virus has now reached its 40th passage. It is still pathogenic for calves.

Titration of embryos infected by way of the yolk sac indicated a virus content of 1 minimal infective calf dose per 0.1 mg. of embryo. This titer compared closely with that of spleen or lymph nodes from rinderpest-infected calves and it was hoped that infected chicken embryos might be substituted for infected bovine tissues in vaccine production. The attempted utilization of egg culture virus in vaccine preparation will be considered in detail in a subsequent report.

THE DISTRIBUTION OF RINDERPEST VIRUS IN EGGS INOCULATED BY WAY OF THE YOLK SAC

After establishing the fact that rinderpest virus could be made to infect chicken embryos regularly, when inoculated by way of the yolk sac, it became of interest to determine the further distribution of the virus in such eggs. It seemed possible that the virus might disseminate from the infected embryo and get into the egg fluids or spread directly to the embryonic membranes which we already knew to be highly susceptible to direct infection.

Seven-day embryonated eggs were inoculated into the yolk sacs in the usual way and, after four days' further incubation, the embryos, membranes, and fluids were harvested separately. The membranes and embryos were washed in three changes of salt solution prior to suspension in salt solution. Then each material was tested separately for virus by inoculation into a susceptible calf. All 3 calves came down with rinderpest, indicating that virus had been present not only in the embryos but in the membranes and fluids as well. This widespread distribution of virus in eggs inoculated by way of the yolk sac has been repeatedly confirmed. We have encountered no instance in which only one particular portion of the egg contained virus without other portions being infected also; that is, if virus was demonstrable in the embryos, it was present also in the fluids and membranes. In only one experiment have we tested yolk sac for the presence of virus

and, as might be anticipated, it was demonstrable.

The exact virus content of the mixed chorioallantoic and amniotic membranes from yolk sac-inoculated eggs has not been accurately titrated but, in one experiment, 1 cc. of a 1:1,000 dilution of membranes produced rinderpest infection in a calf, indicating at least 1 minimal infective calf dose per milligram of membrane. The mixed allantoic and amniotic fluids have been titrated and found to contain 1 minimal infective calf dose of virus per 0.0001 cc. or 10,000 m.i.d.'s per cubic centimeter. Such fluids, though highly infective, are usually water clear, especially if they have been harvested after a preliminary chilling of the eggs. Virus is abundantly present in both the allantoic and amniotic fluids harvested separately and, to judge by the similar incubation periods in calves inoculated with 0.01-cc. doses of either fluid, the virus contents of the two fluids are comparable in amount.

The finding that virus is so widely distributed in eggs inoculated by way of the yolk sac has furnished us an abundance of richly infectious material with which to try to prepare a satisfactory rinderpest vaccine from eggs.

THE EFFECT OF RINDERPEST VIRUS UPON INFECTED EMBRYOS

So far as can be told by gross examination of infected embryos, rinderpest virus exerts little or no deleterious effect. It is true, however, that in every serial egg passage of the virus, quite a large number of the embryos in each group of inoculated eggs succumb on from the second to the fourth day of infection. The precise rôle of the virus in causing these deaths has not yet been thoroughly studied. In eggs incubated beyond the usual four days after inoculation, deaths continue to take place up until the time of hatching, but some eggs do actually hatch and the chicks from such eggs appear outwardly to be quite normal. In embryos inoculated with virus by way of the yolk sac, virus is demonstrable by calf inoculation at any time up until and even after hatching. The question of the persistence of virus in hatched chicks will be considered in detail in a subsequent report. While the exact rôle played by the virus in causing deaths observed in infected embryos cannot at present be appraised, it may be stated with certainty that embryos can be heavily infected with virus and still survive and present a normal appearance grossly.

THE INFLUENCE OF THE EMBRYONATION AGE OF EGGS AT TIME OF INOCULATION UPON THE VIRUS CONTENT OF THEIR FLUIDS FOUR DAYS AFTER INFECTION

The choice of 7-day embryonated eggs for inoculation and a further period of four days' incubation before harvesting was dictated largely by technical considerations and by the desire to obtain as large an amount of infected egg material as possible. The yolk sac becomes progressively smaller as embryonation progresses and, in eggs that have incubated for longer than seven days, it becomes increasingly difficult to inject it, with certainty, by the relatively "blind" method of inoculation that we practice. The allantoic and amniotic sacs, and hence the amount of allantoic and amniotic fluids, increase up to about the eleventh or twelfth days of incubation. These two considerations largely determined our procedure in the first instance of inoculating at seven days' embryonation and harvesting at eleven days. Subsequent study of virus multiplication in eggs of various embryonation ages and in eggs harvested at varying times after inoculation have tended to justify our original choice of procedure.

Groups of eggs embryonated for from five to nine days at time of infection have been inoculated with rinderpest virus by way of the yolk sac. After four days' further incubation at 38 C., the mixed allantoic and amniotic fluids from each group of eggs have been tested for the presence of virus by calf inoculation. One cubic centimeter of undiluted fluid was the test dose administered to calves and the approximate amount of virus was judged roughly by the speed with which the inoculated calves sick-

ened. While the length of the incubation period in calves is not an absolute criterion for use in judging the virus content of materials being tested, it does give a relative approximation. For instance, we know from experience gained in titration experiments that, with comparable materials, an incubation period of two days indicates the presence of a very great deal more virus than does an incubation period of either four or five days. In like manner, an incubation period of three days usually indicates the presence of more virus than does one of four days, just as one of two days usually indicates more virus than does one of three days.

The results obtained in testing fluids from eggs infected at varying embryonation ages are given in table 1.

TABLE 1—The Influence of the Embryonation Age of Eggs at Time of Inoculation via the Yolk Sac upon the Virus Content of the Egg Fluids after Four Days of Infection

Embryonation age of eggs at inoculation	Test for presence of rinderpest virus in egg fluids harvested after four days' infection		
	Result of calf inoculation		
	Calf No.	Result	Incubation period in calves
5 days	141	No illness	
5 days	172	Rinderpest	5 days
6 days	173	Rinderpest	2 days
7 days	139	Rinderpest	2 days
7 days	160	Rinderpest	3 days
7 days	207	Rinderpest	2 days
8 days	189	Rinderpest	4 days
8 days	161	Rinderpest	3 days
9 days	190	Rinderpest	3 days
9 days	162	Rinderpest	4 days

As is shown in the table, tests of the fluids from 2 groups of eggs inoculated when embryonated five days indicated the presence of very little virus in the 1 group whose fluids produced infection in a calf only after an incubation period of five days and none at all in the other group whose fluids failed to infect a calf. The fluids from the group of eggs inoculated when embryonated six days as well as those from the 3 groups of eggs inoculated at seven days contained an abundance of virus and infected the test calves promptly with rinderpest. The fluids of the 8- and 9-day embryonated eggs appeared to contain less virus as judged by the lengthened incubation periods in the test calves. So far as these experiments can be interpreted, it would seem that eggs embryonated for six or seven days at the time of inoculation are the most favorable for use in the cultivation of rinderpest virus.

THE TIME OF APPEARANCE OF VIRUS IN 7-DAY EMBRYONATED EGGS INOCULATED VIA THE YOLK SAC

Since it was apparent from the results outlined above that eggs embryonated seven days at the time of inoculation were satisfactory for use in the cultivation of rinderpest virus, eggs of this embryonation age were used in further experiments designed to learn the time at which virus, introduced by way of the yolk sac, invaded the embryos and fluids. The question has been considered in 4 separate culture series and the results obtained are outlined in table 2.

The results of the first culture series seemed to indicate that the rapidity with which virus spread from the yolk sac to the embryos and fluids was related to the number of serial egg passages to which the virus had been previously subjected. In the nineteenth egg passage, the first of this series to be tested, virus had not yet invaded the embryos forty-eight hours after administration by way of the yolk sac. It had, however, reached the embryos by seventy-two hours and was present at ninety-six hours. The next passage in this culture series to be studied was the 28th, and here fluids instead of the embryos were tested for the presence of virus. As in the nineteenth passage, virus given by way of the yolk sac was present in the fluids only seventy-two and ninety-six hours after inoculation. It had not yet invaded the fluids at twenty-four and forty-eight hours. The 33rd passage was the next in this culture series to be studied and here, in contrast to the earlier passages, it was found that virus already had invaded the fluids and embryos twenty-four hours after inoculation. It was present also in the materials harvested at forty-eight and seventy-two hours. In the 36th passage also, virus had already invaded the fluids twenty-four

hours after administration by way of the yolk sac. From consideration of this culture series alone, it seemed that, sometime between the 28th and the 33rd yolk sac passages, the pathogenicity of the virus had been enhanced to such an extent that, instead of requiring seventy-two hours' sojourn in the yolk sac before it invaded the embryos and extra-embryonic fluids, it invaded in less than twenty-four hours. The change appeared to be one of adaptation and seemed to constitute an intensification in the virulence of the virus for eggs. The change, however, whatever its character, was not accompanied by a detectable increase in the amount of virus in the fluids; the minimal infectious calf dose of fluids after the apparent enhancement of virulence of the virus for eggs did not exceed 0.0001 cc., the same approximate titer found before the change had occurred.

In the second culture series, the time of appearance of virus was studied only in fourth passage eggs. The findings coincided with the view, suggested by the results of study of the first culture series, that virus but recently transferred to eggs spread from the yolk sac to the embryos and fluids more slowly than did virus that had been subjected to prolonged serial passage in eggs. In these fourth passage eggs, virus had not yet invaded the embryos or fluids forty-eight hours after its introduction by way of the yolk sac, but it was present at seventy-two hours.

In the third culture series with sixteenth egg-passage virus, we had expected results similar to those obtained with the second culture series. However, instead, the fluids already contained virus only twenty-four hours after yolk sac inoculation.

The fourth culture series with 38th egg-passage virus was also something of an exception to what might have been anticipated from consideration of the findings in the first culture series. Virus had not yet invaded the egg fluids twenty-four hours after yolk sac inoculation though it was present, probably in low titer, to judge from the long incubation period in the test calf, namely forty-eight hours.

To have been completely in agreement with the view, originally suggested by the findings with the first culture series eggs,

TABLE 2—The Time of Appearance of Rinderpest Virus in the Embryos and Fluids of 7-Day Embryonated Eggs Inoculated via the Yolk Sac

Culture series	Egg passage of virus	Period of incubation of infected eggs	Egg material tested	Tests for infectivity in calves		
				Calf No.	Result	Incubation Period
1	19th	48 hours	Embryos	96	No illness	
	19th	72 hours	Embryos	87	Rinderpest	2 days
	19th	96 hours	Embryos	101	Rinderpest	2 days
	28th	24 hours	Fluids	157	No illness	
	28th	48 hours	Fluids	158	No illness	
	28th	72 hours	Fluids	159	Rinderpest	3 days
	28th	96 hours	Fluids	160	Rinderpest	3 days
	33rd	24 hours	Fluids	209	Rinderpest	3 days
	33rd	24 hours	Embryos	210	Rinderpest	2 days
	33rd	48 hours	Fluids	196	Rinderpest	2 days
	33rd	48 hours	Embryos	197	Rinderpest	2 days
	33rd	72 hours	Fluids	169	Rinderpest	2 days
	33rd	72 hours	Embryos	188	Rinderpest	2 days
	36th	24 hours	Fluids	224	Rinderpest	5 days
	36th	48 hours	Fluids	213	Rinderpest	3 days
	36th	72 hours	Fluids	208	Rinderpest	2 days
2	4th	24 hours	Fluids	163	No illness	
	4th	48 hours	Fluids	169-A	No illness	
	4th	48 hours	Embryos	176	No illness	
	4th	72 hours	Fluids	171	Rinderpest	2 days
	4th	72 hours	Embryos	177	Rinderpest	2 days
	4th	96 hours	Fluids	207	Rinderpest	2 days
3	16th	24 hours	Fluids	265	Rinderpest	2 days
	16th	48 hours	Fluids	276	Rinderpest	3 days
4	38th	24 hours	Fluids	281	No illness	
	38th	48 hours	Fluids	281-A	Rinderpest	5 days
	38th	72 hours	Fluids	274	Rinderpest	3 days

that increased rapidity of invasion of the embryos by virus deposited in the yolk sac was related entirely to the number of egg passages to which the virus had been submitted, the findings in the third and fourth culture series should have been just reversed. What seems more likely from consideration of all the data at hand is that, while the change is influenced by serial egg passage of the virus, it is not one which takes place after any uniform number of passages. Rather, other factors, in addition to mere serial passage, play a rôle, and in different series of cultures, the influence of these other fatcors varies so that in some series the change may take place as it did in our first series at between the 28th and 33rd passage, while in other series the change may have already been effected at the sixteenth passage as it was in our third series of cultures. The point is not of any apparent practical importance, but it does have a theoretical interest in that the increase in rapidity of invasiveness of virus on egg passage probably constitutes actual tangible evidence of adaptation of the virus to eggs.

THE MINIMAL INFECTIVE DOSE OF RINDERPEST VIRUS FOR EGGS BY YOLK SAC AND CHORIOALLANTOIC MEMBRANE INOCULATION

In view of the possibility that certain of the infective egg materials might be made to serve in the production of rinderpest vaccine and that then eggs in large numbers would have to be inoculated, it became of interest to learn something concerning the minimal infective dose of virus for eggs in order that inoculum might be economized.

In order to determine the minimal infective dose for eggs inoculated on the chorioallantoic membranes, groups of 10-day embryonated eggs were inoculated onto the membranes through a window in the shell with 0.2-cc. doses of tenfold dilutions of thirteenth membrane-passage virus. After three days' incubation, the membranes of each group of eggs were harvested separately and tested for the presence of rinderpest virus by calf inoculation. It was found that the membranes from eggs that had received 0.2 mg. or more of infective membrane produced rinderpest in the test calves, while eggs that had received 0.02 mg. of infective membrane were not infective for calves. It appeared from this that the m.i.d. of infected membranes for eggs inoculated on the chorioallantoic membranes was approximately 0.2 mg.

To determine the m.i.d. for eggs inoculated by way of the yolk sac, groups of 7-day embryonated eggs were inoculated into the yolk sac in the usual manner with 0.5-cc. doses of tenfold dilutions of fifteenth membrane-passage virus. After four days' incubation, the embryos of each group of eggs were harvested separately and tested for the presence of virus by the inoculation of susceptible calves. It was found that the embryos from eggs that had received 0.5 mg. or more of infective membrane produced rinderpest in the test calves, while the embryos from eggs that had received 0.05 mg. of infective membrane were not infective for calves. It appeared from this that the m.i.d. of infected membranes for eggs inoculated *via* the yolk sac was approximately 0.5 mg.

It is unfortunate that the quantity of inoculum for eggs by both routes was not kept the same instead of using 0.2-cc. doses for membrane and 0.5-cc. doses for yolk sac inoculation, because in all probability the doses of virus necessary to infect eggs by either route are roughly identical. The m.i.d. found for eggs is also in approximate accord with the usual calf m.i.d. of 0.1 mg. of either membranes or embryos.

UNSUCCESSFUL ATTEMPTS TO ESTABLISH RINDERPEST VIRUS IN EMBRYONATING EGGS BY YOLK SAC INOCULATION OF BOVINE SPLEEN VIRUS

Following the discovery that chicken embryos and the extra-embryonic fluids could be regularly infected with egg-established rinderpest virus administered by way of the yolk sac, it became of interest to learn whether one could infect embryos and fluids by this route directly with calf-pass-

age virus which had had no preliminary adaptive passages on the chorioallantoic membranes. It seemed possible that our current success in infecting by way of the yolk sac might in some way be accounted for on the basis of our use of virus that had become egg-adapted by virtue of a series of preliminary passages on the chorioallantoic membrane. Conversely, our earlier series of failures to transmit rinderpest virus serially in eggs by yolk sac inoculation might have resulted more from the actual failure of bovine-passage virus to adapt to eggs by this route of inoculation than to what, at first sight, had seemed the unfortunate choice of yolk sac instead of embryos or fluids as the inoculum at each serial passage.

In order to further study the possibilities, a group of 7-day embryonated eggs were inoculated by way of the yolk sac with a suspension of spleen from a calf ill of rinderpest. The embryos, when harvested four days later, were found to have become infected with rinderpest virus as demonstrated by calf inoculation. The serial passages were continued in eggs, using embryos and fluids of one passage to inoculate the eggs of the succeeding passage by way of the yolk sac. Embryos of the fourth serial egg passage were tested for virus by calf inoculation. They failed to infect, indicating that the virus had been lost sometime subsequent to the first egg passage.

Instead of reverting to the first passage in attempting to continue the series, a second group of egg cultures was started with infected calf spleen. Embryos of the first passage proved infective for a calf but by the third passage the virus had been lost.

A third group of egg cultures was started, as before, with bovine spleen virus. This time, embryos of the first and second passage proved infective for calves and the titer in the second passage was the same as that already recorded for embryos of more advanced egg passages, namely, 1 minimal infective calf dose per 0.1 mg. of embryo. The virus was, however, lost by the fourth passage.

A fourth group of egg cultures was initiated and, again, embryos of the first passage infected a calf. At the fourth serial passage also, virus was demonstrable in the fluids and embryos by calf inoculation. This series of cultures was not tested again for virus until the tenth passage, and by this time the virus had once more been lost.

These failures to maintain rinderpest virus from bovine spleen through more than a few serial passages in eggs by yolk sac inoculation contrasted strongly with the ease with which cultures of the virus from the same source could be initiated and maintained on the chorioallantoic membrane. They also contrasted with the ease with which we could maintain, by serial yolk sac passage, strains of the virus that, though initially derived from calf spleen, had been submitted to a series of preliminary passages on the chorioallantoic membrane. It seemed quite apparent that, though rinderpest virus, direct from calf spleen, was capable of infecting embryos and fluids when administered by way of the yolk sac, it could not be maintained indefinitely in serial passage by this route of administration. Sometime after its first serial passage, and probably by its fifth serial passage, it lost its capacity either to survive or multiply in the egg when introduced by way of the yolk sac. There seems little doubt that actual multiplication of the virus occurred for a while at least because in one instance the titer of the infected embryos of the second serial passage was the same as that of the infected calf spleen used in initiating the series of cultures. It was obvious that some adaptive change in the virus was required before it could maintain itself indefinitely in embryos and fluids when administered by way of the yolk sac. This adaptive change, whatever its character, could be effected by a preliminary series of transfers on the chorioallantoic membranes of embryonating eggs.

In order to obtain information concerning the number of chorioallantoic passages required to endow the virus with the capacity to multiply indefinitely in the embryo when administered by way of the yolk sac, the following experiment was carried out: 4 samples of egg-passage rinderpest virus

that had been transferred 4, 8, 12, and 16 serial passages on the chorioallantoic membrane were each administered by way of the yolk sac to 4 different groups of 7-day embryonated eggs. The egg cultures thus started with viruses of differing membrane passages were then continued in the usual way, transferring mixed allantoic-amniotic fluids of each passage to the yolk sac of the eggs of the succeeding passage. At the third serial yolk sac passage, fluids from each group of eggs were tested for the presence of virus by calf inoculation. The fluids from the cultures that had been initiated with fourth and eighth passage membrane virus failed to infect calves while those initiated with twelfth and sixteenth membrane-passage virus produced rinderpest in the test calves. From this experiment, it may be concluded that, at sometime between the eighth and twelfth serial chorioallantoic membrane passages, the virus under study had become adapted to eggs sufficiently to be then capable of serial transmission by way of the yolk sac. The nature of this change effected in rinderpest virus by serial membrane passage is not clear from the data at hand. That it does not involve the acquisition of invasiveness for the embryo is precluded by the fact that, even in the first passage by way of the yolk sac, bovine virus is capable of invading the embryo. Rather, it would seem that the virus of direct bovine origin possesses only a limited amount of something which enables it to multiply when administered *via* the yolk sac, that it produces no more of whatever the unknown hypothetical factor is and that, once the factor is exhausted, multiplication of the virus in the egg no longer takes place. Bovine virus, however, placed on the chorioallantoic membrane for a series of passages, not only multiplies of itself but appears to acquire the capacity for producing more and indefinitely of the hypothetical factor essential for transmission of the virus by the yolk sac. It would seem that between 8 and 12 serial passages of the virus on the egg membranes are required to "educate" it to the production of the requisite factor.

IMMUNOLOGIC EVIDENCE OF THE IDENTITY OF EGG-CULTIVATED VIRUS WITH THAT FROM INFECTED BOVINE TISSUES

Although the disease produced in calves by inoculation with virus that has been cultivated in eggs has all the clinical and pathologic characteristics of that similarly induced by rinderpest virus that has been transferred only in calves, the possibility that the egg-cultivated virus might be other than rinderpest virus exists. Since we had the means at our disposal of removing any doubts that there might be on this score, it was decided to establish as firmly as possible the claim that the virus grown in eggs was actually rinderpest virus. There are four types of immunologic evidence which bear on the point and all indicate the identity of the egg-passage virus with the bovine-passage virus. These are as follows:

1) Serum from a calf, recovered from infection with egg virus, neutralized bovine-passage virus in calves. Conversely, serum from a calf recovered from infection with bovine virus neutralized egg-passage virus in calves.

2) Calves recovered from infection with egg-passage virus were immune to bovine-passage virus.

3) Calves vaccinated, as outlined in Interim Report 3,[2] with chloroformized tissue suspensions from calves infected with egg-passage virus were solidly immunized against bovine-passage virus.

4) The serum of rabbits, immunized by multiple intraperitoneal injections of egg-passage virus, neutralized bovine-passage virus in calves.

The findings in these 4 groups of immunologic experiments leave no doubt that the virus we have grown in eggs is actually identical with the one we are transmitting in calves as rinderpest virus.

DISCUSSION

Various routes of inoculating embryonating eggs have been tried in an attempt to establish rinderpest virus in embryos and in the extra-embryonic fluids. Intra-allantoic or intra-amniotic injection of the virus

proved, unsuccessful. However. it has been found that, under certain conditions, administration of virus by way of the yolk sac results in infection not only of the embryos but of the membranes and extra-embryonic fluids as well. The virus titers attained in all infected portions of the egg are reasonably high. However, what appears to be an actual adaptation of the virus must take place before it can be regularly transmitted by way of the yolk sac and the adaptation seems to take place in two stages.

If virus of bovine origin is administered directly to 7-day embryonated eggs by way of the yolk sac, it is found to be present four days later in both the embryos and the extra-embryonic fluids. However, it can apparently be transferred in series from this original infectious material, at the most, for only a few further serial passages by way of the yolk sac before it is lost. In order to get the rinderpest virus into a state in which it is capable of being serially transferred indefinitely by way of the yolk sac, the first of the two adaptive stages mentioned previously must be effected. In our experiments, this was achieved by a series of preliminary chorioallantoic membrane passages of virus in 10-day embryonated eggs. While the number of membrane passages required to complete the adaptation is probably influenced by some factors that are not completely controllable, in one experiment in which we studied the question, it was found that 4 and 8 serial membrane passages were insufficient to achieve the adaptation, but that after 12 and 16 serial membrane passages, the virus was capable of further serial transmission by way of the yolk sac.

The second stage in the adaptation of rinderpest virus to satisfactory transmission by way of the yolk sac takes place after the first stage has been accomplished. It consists in a shortening of the period elapsing between the time of introduction of the virus into the yolk sac and its eventual invasion of the embryos and fluids. In cultures in which the second adaptive change has not yet occurred, the virus, after introduction into the yolk sac, does not invade the embryos and fluids until something over forty-eight hours later. By seventy-two hours after injection it is, however, present in both the embryos and fluids. After completion of the second stage of adaptation, the virus, injected by way of the yolk sac, invades the embryos and extra-embryonic fluids promptly and is present there within twenty-four hours of the time administered.

The time required to achieve the second adaptive change is variable and appears not to be solely dependent upon the number of serial egg passages to which the virus has been subjected. In one culture series, the change occurred sometime between the 28th and 33rd serial passage; in another it was already complete at the sixteenth passage, while in a third series it was apparently not yet complete at the 38th serial passage. The change is seemingly one of increased pathogenicity of the virus for embryonic tissues and is unaccompanied by any detectable increase in the titer of virus in the infected embryos or fluids.

Our procedure in passaging the virus serially in eggs by yolk sac inoculation is not influenced by whether or not the second adaptive change has occurred because we routinely permit infected eggs to incubate for at least seventy-two and usually ninety-six hours before transferring. The technique employed in transferring is briefly as follows: After incubating at 38 C. for seventy-two or ninety-six hours following administration of virus by way of the yolk sac, the infected eggs are chilled by placing them in a refrigerator (at ± 2 C.) with their air sac ends uppermost. When they have cooled for approximately four hours, the shell at the air sac end is removed aseptically, the shell, chorioallantoic and amniotic membranes are reflected with forceps and scissors, and the mixed allantoic and amniotic fluids are removed by pipette using a rubber bulb for suction. Ordinarily, only the fluids are harvested for virus to be used in passage. If the embryos are to be saved for virus, they are removed aseptically with forceps and scissors. For use in infecting eggs of the next serial

passage, the fluid is usually diluted with 3 or 4 parts of sterile physiologic salt solution before injection into the next group of eggs. If the embryos are to be used for passage virus, they are decapitated, weighed, and ground in a Ten Broeck pyrex glass grinder with physiologic saline to make a 10 per cent suspension. For inoculation of the next serial passage, 7-day embryonated eggs are placed in racks with their air sac ends uppermost after candling and marking the position of the embryo. A small hole is drilled in the air sac end and through this the yolk sacs are inoculated with 0.5 cc. of either the diluted egg fluids or the 10 per cent embryo suspension of the preceding passage. For inoculating, a 1-in., 21-gauge needle on a 10-cc. Luer syringe is used.

The infected fluids or the embryo suspensions may be kept for a matter of weeks, stored frozen at —6 F., without loss of infectivity. It is our practice, when large groups of eggs are to be infected, to store the virus intended for use frozen until tests of its bacteriologic sterility have been completed.

Although more deaths occur among infected embryos during the four days after inoculation than take place among similar groups of uninfected eggs during the corresponding period of embryonation, the exact rôle played by the virus in inducing these deaths is not known. Embryos which appear active and of normal appearance grossly may be present in eggs whose fluids contain at least 50,000 minimal infectious calf doses of rinderpest virus, and whose membranes contain at least an additional 2,000 m.i.d.'s. The embryos themselves in such eggs will contain approximately 30,000 m.i.d.'s of virus. Thus, in an egg whose total virus load at the fourth day of infection exceeds 80,000 m.i.d.'s, an apparently normal embryo can and frequently does develop and grow for a number of days. Some will even hatch as apparently normal and active chicks though they continue to carry the virus even after hatching. There is some tendency for the mortality among the embryos of infected eggs to increase with increasing serial passage of the virus. This, however, is not regular or dependable, and quite marked variations in the mortality rates in either direction may be observed between eggs of even two consecutive passages. If rinderpest virus does have a specific lethal effect upon chick embryos, it is too irregular in its occurrence to serve any useful quantitative purpose in research with the virus.

SUMMARY

1) Rinderpest virus introduced into either the allantoic or amniotic sacs of 10-day embryonated eggs failed to infect the embryos.

2) Rinderpest virus of direct bovine origin, injected into 7-day embryonated eggs by way of the yolk sac, infected both the embryos and the extra-embryonic fluids. The virus thus established could not be transmitted indefinitely in series in eggs, however, by yolk sac inoculation.

3) Rinderpest virus that had been subjected to a preliminary series of chorioallantoic membrane passages was capable of being transmitted indefinitely in series in eggs when administered by way of the yolk sac. In such eggs, the virus was widely distributed, being present in the embryos, the extra-embryonic fluids, and the egg membranes. Between 8 and 12 serial chorioallantoic membrane passages were required, in one experiment in which the question was studied, to adapt the rinderpest virus to yolk sac propagation.

4) A second adaptive phase through which the rinderpest virus passed upon serial transfer in eggs by yolk sac inoculation was characterized by a shortening of the time elapsing between introduction of the virus into the yolk sac and its eventual appearance in the embryos and extra-embryonic fluids. In early serial passages of virus by this route, the virus did not reach the embryo, after being placed in the yolk sac, until between forty-eight and seventy-two hours after inoculation. In later passages, after the second adaptive phase was complete, the virus reached the embryos and extra-embryonic fluids within twenty-

four hours of the time it was introduced into the yolk sac.

5) Rinderpest virus appears to have no constant and dependable lethal effect upon chicken embryos, although many infected embryos do succumb.

6) Eggs that have been embryonated for six or seven days at time of inoculation are most favorable for use in the propagation of virus by way of the yolk sac. Five-day embryonated eggs are completely unsatisfactory for use and 8- or 9-day embryonated ones are less satisfactory than those of seven days.

7) The minimal infective dose of rinderpest virus for eggs, either by chorioallantoic membrane or yolk sac inoculation, is roughly in agreement with the subcutaneous m.i.d. of virus for calves.

8) Immunologic evidence indicating the identity of the egg-cultivated virus with rinderpest virus of direct bovine origin is presented.

References

[1]First Interim Report, July 16, 1943.

[2]Third Interim Report, Jan. 6, 1944.

Rinderpest

V. Attempts to Prepare an Effective Rinderpest Vaccine from Inactivated Egg-Cultivated Virus

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AS STATED in a previous report,[1] a rinderpest vaccine prepared after the general method recommended by Kelser[2] has proved efficacious in our hands. This vaccine, made from infected bovine tissues in which the virus is inactivated with chloroform, induces a prompt and satisfactory immunity in cattle after single administrations in dosage as small as 10.0 cc. It can be easily and rapidly prepared, and any other vaccine to replace it would have to possess quite marked superiority in several directions. In our experience with the Kelser vaccine, we have encountered only two unfavorable features, neither of which is of any particularly serious character, but both of which have to do with the source of virus from which the vaccine is prepared.

In the first place, the yield of vaccine from an individual calf is not high. Of the first 132 calves employed by us in vaccine production, the yield of usable tissue per head averaged 1,368 Gm., which constituted, on the basis of 10.0 cc. of vaccine as an immunizing dose, 684 doses of vaccine per head. Secondly, there is always the hazard of including bacterial pathogens dangerous for cattle in a vaccine prepared from bovine tissues.

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The monthly reports occasionally referred to in this publication are official reports on the progress of the project, rendered monthly to the U. S.-Canadian Commission and filed in the Office of the Chief of Chemical Warfare. Interim Reports are special reports on important scientific accomplishments and developments in the project and rendered at such times as developments and circumstances warranted. Most of these Interim Reports constitute the basis of the present publication.

In the hope of avoiding the features found unfavorable in the bovine tissue vaccine and on the possibility that a preparation with superior immunizing potency might be achieved, we have therefore attempted to use egg-propagated rinderpest virus in vaccine production. If egg-grown virus could be substituted for that of infected bovine tissues in the preparation of vaccine, without sacrificing any of the immunizing potency of the finished product, certain advantages from the standpoint of economy of production, safety, and availability in large quantities would accrue. We knew from our work in the cultivation of rinderpest virus in embryonating eggs[3, 4] that the titratable content of virus in both the membranes and the embryos was about the same as that in infected bovine tissues, namely, 1 m.i.d. per 0.1 mg., while that of the egg fluids was 1 m.i.d. per 0.0001 cc. of fluids. An average egg would thus yield sufficient virus to prepare approximately 7 doses of vaccine of similar virus potency to our bovine tissue vaccines and, providing that inactivated egg virus per original minimal infective dose had the same immunizing potency as inactivated bovine virus, quite a saving in cost of vaccine per dose would be effected. From these calculations, it was apparent that from 100 embryonated eggs we might be able to produce the equivalent in vaccine that, using bovine tissue as source, required 1 calf. Furthermore, the use of eggs instead of calves for virus propagation avoided the necessity for the time- and labor-consuming procedures required in maintaining isolation quarters and facilities for large animals.

Unfortunately, egg-propagated virus has been found to be ineffective, after inactivation with either chloroform or formalin, in immunizing cattle against rinderpest. Of 31 calves vaccinated with various preparations of inactivated egg-propagated virus, not a single one has proved solidly immune to a subsequent challenge dose of rinderpest virus. Usually, a variable degree of partial protection is achieved by the egg vaccines, but in no instance has it been the complete type of immunity regularly induced by bovine tissue vaccines.

EXPERIMENTAL

Egg virus vaccines have been prepared from the mixed chorioallantoic and amniotic membranes of eggs infected by way of the chorioallantoic membranes[3] and from the embryos, mixed allantoic and amniotic fluids, and the yolk sacs of eggs infected by yolk sac inoculation.[4] Eggs from either Barred Plymouth Rock or White Leghorn hens were used. Membrane-cultivated virus was harvested for vaccine production on the third day of propagation. The membranes were ground and suspended in physiologic saline to make 20 per cent by weight suspensions prior to adding the inactivating agent. The virus in eggs infected by way of the yolk sac was ordinarily permitted to propagate for four days before the embryos, fluids, or yolk sacs were harvested for vaccine production. The embryos and yolk sacs were ground and suspended in

TABLE I—Attempted Immunization against Rinderpest with Formalin- and Chloroform-Inactivated Egg-Propagated Virus Vaccines

Calf No.	VACCINE		IMMUNIZATION		**Challenge infection with 100-1,000 m.l.d.'s rinderpest virus (bovine spleen)
	Source of egg virus	Inactivated with	Dose of vaccine/-injection	No. of subcutaneous injections	
39	Membranes*	Chloroform .75%	5.0 cc.	3	Positive—sick 7 days—recovered
40	Membranes	Chloroform .75%	7.5 cc.	3	Positive—sick 8 days—recovered
156	Fluids	Chloroform .75%	25.0 cc.	1	Positive—sick 4 days—recovered
167	Fluids	Chloroform .75%	15.0 cc.	3	Positive—sick 6 days—recovered
168	Fluids	Chloroform .75%	25.0 cc.	3	Positive (died 13th day)
283	Embryos	Formalin—1 in 1,000	30.0 cc.	2	Positive (died 14th day)
284	Embryos	Chloroform .75%	30.0 cc.	2	Positive—sick 9 days—recovered
285	Fluids	Chloroform .75%	30.0 cc.	2	Positive (died 8th day)
286	Fluids	Formalin—1 in 1,000	30.0 cc.	2	Positive—sick 8 days—recovered
290	Fluids	Chloroform .75%	30.0 cc.	2	Positive (died 8th day)
291	Yolk sacs	Chloroform .75%	30.0 cc.	2	Positive (died 9th day)
69		Control (not vaccinated)			Positive (died 6th day)
155		Control (not vaccinated)			Positive (died 7th day)
211		Control (not vaccinated)			Positive (died 7th day)
308		Control (not vaccinated)			Positive (died 8th day)

*In preparation of egg virus vaccines, the membranes, embryos, and yolk sacs were ground and suspended in physiologic saline to make 20% by weight suspensions. The fluids were used undiluted.
**Challenge dose of virus administered 14 days after last vaccine injection.

physiologic salt solution to make 20 per cent suspensions before adding the inactivating agent, while the fluids were used undiluted and the inactivating agents added directly. In certain experiments, virus propagated for longer or shorter periods of time in eggs was used in the preparation of vaccines but these were no more effective in inducing immunity than were those made with virus grown for three or four days. Formalin, 1:1,000, or 0.75 per cent chloroform were used to inactivate the egg-propagated virus in the vaccines prepared.

Since we had set the bovine tissue vaccine as our criterion for an acceptable vaccine, most of the immunization experiments with inactivated egg-propagated virus vaccines were terminated when it became clear that the vaccines under test were not going to be effective in inducing a complete immunity to the challenge infection with rinderpest virus. In certain experiments, however, the animals were left under observation throughout their entire course. A composite table of the findings in such experiments is given in table 1.

As can be seen from the table, none of the 11 vaccinated animals included were completely immune to the challenge dose of rinderpest virus. Five died of rinderpest, though as a rule they survived longer after challenge than did the 4 control calves, all of which also died. The remaining 6 vaccinated calves underwent rinderpest attacks of varying severity and eventually recovered. In 4 of these recorded in the table as sick for from seven to nine days, it was "touch and go" for several days as to whether they would eventually die or recover. They were emaciated and in miserable shape for a number of days after they began to recover. The remaining 2 vaccinated animals, Calves 156 and 167, febrile for four and six days, respectively, after challenge, were not seriously ill of rinderpest and made prompt recoveries.

Even though it is quite evident from consideration of the results just outlined that the egg virus vaccines had afforded some degree of protection against rinderpest, it was also evident that the degree of this protection was completely inadequate to be of any practical value for use in the field. The egg virus vaccines did not even closely approach the bovine tissue vaccines in effectiveness.

The only point of apparent significance to be derived from the results shown in table 1 is that, seemingly, the calves that acquired the most effective degree of immunity from inactivated egg virus vaccine were those receiving the smallest total amounts of vaccine. This suggested that, in the egg vaccines, two opposing or antagonistic factors were at work. One of these was the inactivated virus *per se* which, from studies with the bovine tissue vaccines, we knew should induce immunity. The other was some factor or substance which prevented the virus from exercising its immunizing effect. In egg vaccines, the two factors appeared to be in sufficient imbalance so that, if the dosage of vaccine was diminished sufficiently, a point could be reached where the amount of the interfering factor was inadequate to exert its complete antagonistic effect. It is believed that the chick or egg protein, contained in the egg vaccines, is the interfering substance and that it acts by preoccupying the antibody-forming mechanism of calves to which the egg vaccines are administered. Thus, when calves are injected with inactivated egg-propagated rinderpest virus, actually at least 2 antigens are administered. One of these is the inactivated virus and the other is the chick or egg protein. These 2 antigens compete for the immunity response and the latter antigen, being predominantly preponderant, preoccupies the calf's antibody-forming mechanism so completely that only a very minor response to the virus protein is elicited. If massive doses of egg vaccine are given, the preoccupation of the calf's antibody-forming mechanism by chick or egg proteins is more complete, and the immunity to rinderpest virus is thus less effective, than if smaller doses of egg vaccine are administered.

If this hypothesis is actually a tenable one, egg vaccine should also interfere with the immune response of calves to known effective bovine tissue rinderpest vaccine when the 2 are administered in mixture. To

test this possibility, egg-propagated virus vaccine was mixed with bovine tissue vaccine and used in an attempt to immunize a calf. The result is shown in table 2.

As shown in the table, Calf 192, which received a single injection of 25.0 cc. of a mixture containing 10.0 cc. of chloroformized bovine tissue vaccine (batch 1) and 15.0 cc. of chloroformized chicken embryo and membrane vaccine (batch 4) was not immunized against rinderpest. When challenged two weeks after the vaccination, the calf underwent a severe, though not fatal attack of rinderpest. That the bovine tissue vaccine contained in the mixture was amply capable of immunizing is shown by the results with the other calves included in the table, vaccinated with diminishing single doses of batch 1 vaccine. An amount of this vaccine as small as 3.0 cc. completely immunized, while 1.0 cc. immunized more adequately when given alone than had the 10.0 cc. mixed with egg vaccine. It may be concluded from the results outlined in table 2 that the addition of the egg vaccine diminished the immunizing potency of the bovine tissue vaccine at least tenfold. That this deleterious action was not peculiar to substances from infected eggs was shown later by the fact that a suspension of normal, uninfected chicken embryo exercised a similar effect upon the immunizing potency of bovine tissue vaccine. It is believed that these findings are in accord with the hypothesis mentioned earlier, that chick or egg proteins, contained in egg vaccines, so completely block out or preoccupy the antibody-forming mechanism that calves to which the vaccines are administered react only very inadequately to the virus antigen also contained in the vaccine.

We have tried various means of separating the virus from the chick and egg proteins with which it is associated in egg vaccines. When infected egg fluids are thawed from the frozen state, a flocculent precipitate results. Virus is present in the precipitate which can be separated by centrifuging and in the clear supernatant fluid. However, inactivated vaccines, made from either the precipitate or supernatant, are completely ineffective in immunizing calves to rinderpest. In fact, there is the suggestion that vaccination of calves with frozen egg virus vaccine actually sensitizes them to rinderpest virus.

TABLE 2—Attempted Immunization against Rinderpest with a Mixture of Inactivated Egg Virus Vaccine and Bovine Tissue Virus Vaccine

Calf No.	20% bovine tissue vaccine—batch 1	20% chicken embryo and membrane vaccine—batch 4	Challenge infection with 100-1,000 m.l.d.'s rinderpest virus 2 wks. after vaccination
134	12.5 cc.	Nil	Negative (immune)
192	10.0 cc.	15.0 cc.	Positive—sick 9 days—recovering when killed
135	5.0 cc.	Nil	Negative (immune)
195	3.0 cc.	Nil	Negative (immune)
194	1.0 cc.	Nil	Positive—sick 2 days—recovering when killed
202	Control—not vaccinated		Positive (died 9th day)
142	Control—not vaccinated		Positive (died 9th day)

The addition of chloroform to infected egg fluid in the virus-inactivating process causes a precipitate which appears to be largely protein. Separation of this precipitate by centrifugation yields a clear supernatant presumably freed of the precipitated protein. However, this clear supernatant is no more effective in immunizing calves than is the crude egg fluid vaccine.

The addition of ammonium sulphate to infected egg fluid results in a flocculent precipitate and the precipitate brought down by one-third saturation with this salt contains most of the virus activity. However,

it contains much of the egg protein as well and when inactivated with chloroform fails to yield a vaccine that effectively immunizes against rinderpest.

There is no evidence at all to indicate that the cultivation of rinderpest virus in embryonating eggs alters its antigenic composition in any way or that the virus growing in eggs is immunologically different than that multiplying in the tissues of infected calves. In fact, all of the evidence that can be brought to bear indicates an antigenic sameness of egg and bovine virus. Thus, calves recovered from infection with egg-propagated virus are solidly immune to straight bovine-passage virus and their blood serums neutralize the bovine virus completely. Furthermore, the very best immunizing bovine tissue vaccine we have ever succeeded in producing was made from the tissues of a calf infected with rinderpest virus that had been subjected to 4 serial egg passages. There are thus no grounds for considering the failure of inactivated egg vaccines to immunize as resulting from any lack of antigenicity of the egg-propagated virus itself.

However, to make certain on this point and to assure ourselves that prolonged propagation of the virus in eggs did not, in some obscure manner, render it incapable of immunizing when inactivated with chloroform, the following experiment was carried out. Calf 231 was inoculated with straight bovine-passage virus and Calf 233 with egg-propagated virus of the 37th serial passage. Both animals developed a febrile reaction on the morning of the third day after inoculation. On the morning of the third day of fever, both were sacrificed and subjected to autopsy and chloroform-inactivated vaccines prepared from their tissues in the usual manner[1]. The two vaccines, after sufficient aging, were tested for their capacity to immunize calves against rinderpest in single doses of 5.0 cc. and 10.0 cc. The results are depicted in table 3.

As can be seen from the table, neither vaccine protected completely in 5.0-cc. dosage, but both protected when single doses of 10.0 cc. were used prophylactically. So far as can be concluded from this experiment then, rinderpest virus that had been subjected to 37 consecutive serial passages in embryonating eggs made just as satisfactory an immunizing agent as did virus of continuous bovine passage, when inactivated with chloroform. It is perfectly apparent from this experiment that the failure of egg virus vaccines to immunize satisfactorily against rinderpest is not the result of any antigenic alteration in the virus itself. Rather, the success or failure of egg-grown virus to immunize is determined by the menstruum in which it is at the time it is inactivated in the process of vaccine production. If it is in the homologous bovine tissues, it makes an effective product, but if it is in a heterologous medium of egg fluids or chicken embryo constituents, it makes a poor vaccine. The failure of the egg vaccine is not due to any lack of antigenicity of the inactivated virus contained, but rather to interference with

TABLE 3—Immunization against Rinderpest with Chloroformized Bovine Tissue Virus Vaccine Prepared from Calves Infected with Bovine-Passage and Egg-Passage Rinderpest Virus

	IMMUNIZATION		
Calf. No.	Source of tissues for vaccine	Amount of vaccine given	Challenge infection with 100-1,000 m.i.d.'s rinderpest virus 2 wks. after vaccination
241	Calf 231—Infected with bovine-passage virus	5.0 cc.	Positive (mild rinderpest)
268	Calf 231—Infected with bovine-passage virus	10.0 cc.	Negative (immune)
242	Calf 233—Infected with 37th egg-passage virus	5.0 cc.	Positive (mild rinderpest)
269	Calf 233—Infected with 37th egg-passage virus	10.0 cc.	Negative (immune)
279	Control—not vaccinated		Positive (died 9th day)

the immune response to it by the heterologous protein menstruum with which it is associated in the vaccine.

Discussion

We have been unsuccessful in preparing a satisfactory rinderpest vaccine with virus as propagated in eggs. Our inactivated egg vaccines have not, in a single instance, induced immunity of the complete and solid type that one regularly achieves with inactivated bovine tissue vaccines. Quite frequently, however, the egg vaccines have conferred upon calves to which they were administered, a partial immunity sufficient to protect against death but not against clinical illness.

So far as we have been able to determine, the failure of inactivated egg virus vaccines to protect satisfactorily against rinderpest is not due to any modification or diminution in the specific antigenicity of the virus they contain. Rather it would seem to result from interference or blocking out of the specific immune response to the virus by other substances contained in the egg vaccine. It is believed that these interfering substances are embryo or egg proteins which, predominating in the vaccine, so completely preoccupy the antibody-forming mechanism of calves to which administered as to seriously hamper an adequate immune response to the inactivated virus itself.

Summary

1) Chloroform- or formalin-inactivated vaccines prepared from rinderpest virus present in the membranes, yolk sacs, embryos, or fluids of infected embryonating eggs failed to induce a satisfactory immunity in calves against rinderpest.

2) Mixture of a suspension of either infected or normal chicken embryo with known effective bovine tissue vaccine markedly decreased the immunizing effectiveness of the bovine vaccine.

3) Tissues from a calf infected with rinderpest virus that had been subjected to 37 serial passages in eggs, when prepared into chloroformized tissue vaccine, made a preparation that immunized satisfactorily and completely against rinderpest.

4) It is believed that the evidence presented points to the likelihood that the failure of inactivated egg virus vaccines to immunize against rinderpest is not due to any lack of immunizing potency of the virus they contain, but rather to the presence of interfering embryo or egg proteins in the preparations.

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[1]Third Interim Report, Jan. 6, 1944.

[2]Kelser, R. A., Youngberg, S., and Topacio, T.: An Improved Vaccine for Immunization Against Rinderpest. J.A.V.M.A., 74, (1929): 28.

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Rinderpest
VI. The Persistence of Virus in Chicks Hatched from Infected Eggs

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MOST viruses that have proved adaptable to propagation in embryonating eggs, and in the study of which eggs have been extensively employed, eventually reach such pathogenicity for the embryo that hatching does not occur. So far as we can learn from the limited literature available to us up here, persistence of a virus in chicks, hatched from eggs infected during embryonation, has not previously been recorded. Certainly it is not a well-known phenomenon at any rate.

In our work with rinderpest virus, we had occasion to permit some infected eggs to embryonate to the eighteenth day. The embryos that were still alive appeared normal and active and it was decided to let those not required immediately for use to continue through the 21st day. From about 50 of these eggs that contained live embryos on the eighteenth day, 3 chicks eventually hatched on the 21st day. The poor hatch was probably, at least partly, due to the fact that the eggs were in a bacteriologic type of incubator and under unfavorable conditions for hatching. Of the 3 chicks that hatched, 1 had a partially uninvaginated yolk sac and was destroyed. The remaining 2 chicks appeared essentially normal and were kept under observation until they were 3 days old. They were then killed and subjected to autopsy, and their brains and solid viscera ground and suspended in physiologic saline preparatory to testing for the presence of rinderpest virus. The calf inoculated with the brain and organ suspension from these 2 chicks came down promptly with rinderpest, indicating that virus had persisted in the hatched chicks for three days. The eggs from which the chicks had hatched had been inoculated with rinderpest virus by way of the yolk sac when embryonated seven days. The virus had thus been present in the embryos for approximately the last fourteen days of embryonation and had survived in the chicks after hatching.

Because the phenomenon of virus survival after hatching was in itself interesting and also because of the possibility that it might be of epidemiologic importance, it was decided to study it further.

EXPERIMENTAL

Four other series of eggs were inoculated by way of the yolk sac, with either bovine spleen or egg-passage rinderpest virus in the usual manner.[1] The hatches in all four series of eggs were extremely poor but the exact rôle that the virus played in this is difficult to appraise because, in order to avoid scattering the virus about in places that would be difficult to disinfect adequately, the eggs, during the last week of incubation, were kept in metal pans in an ordinary bacteriologic incubator and were thus under unfavorable conditions for hatching. Normal uninoculated eggs incubated under similar conditions hatched but a slightly higher percentage of chicks than did the infected eggs.

In testing for the presence of virus in the hatched chicks, the viscera or other materials to be studied were made up in

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pools of from 2 to 6 chicks. Twenty per cent suspensions were prepared in physiologic saline and administered in either 3.0-cc. or 6.0-cc. dosage subcutaneously to susceptible calves. The results obtained in testing for the presence of rinderpest virus in hatched chicks are given in table 1.

TABLE 1—The Persistence of Rinderpest Virus in Chicks Hatched from Infected Eggs

Chicks hatched from eggs of series	Age of chicks at time of test for virus	Material tested for virus	Result of calf inoculation
A*	1 day	Brains and solid viscera	Positive (rinderpest)
B	1 day	Solid viscera**	Positive (rinderpest)
C	2 days	Solid viscera	Positive (rinderpest)
C	2 days	Whole blood	Negative (no illness)
D	2 days	Whole blood	Negative (no illness)
E	3 days	Brains and solid viscera	Positive (rinderpest)
A	4 days	Brains	Positive (rinderpest)
A	4 days	Solid viscera	Positive (rinderpest)
D	5 days	Solid viscera	Positive (rinderpest)
A	6 days	Feces	Negative (no illness)
A	8 days	Whole blood	Negative (no illness)
D	8 days	Brains	Negative (no illness)
D	8 days	Solid viscera	Negative (no illness)
C	8 days	Brains	Negative (no illness)
C	8 days	Solid viscera	Negative (no illness)
C	11 days	Solid viscera	Negative (no illness)
A	11 days	Brains and solid viscera	Negative (no illness)

*The eggs in series "A" and "C" were inoculated with bovine spleen rinderpest virus, while those in "B", "D", and "E" were inoculated with virus that had been transferred, respectively, 37, 41, and 40 serial passages in eggs.

**Lungs, liver, spleen, kidneys, ovaries or testicles, and adrenals were pooled and included as "solid viscera."

As is shown in the table, virus was present in the chicks hatched from "series A" eggs on the first and fourth days but had disappeared by the eleventh day. It was present in both the brains and the solid viscera, which were tested separately in calves, in the case of the chicks examined when 4 days old. Only a single test for the presence of virus was made in the case of chicks hatched from "series B" eggs and it was found present in the solid viscera at 1 day of age. In the chicks hatched from "series C" eggs, virus was present in the solid viscera of chicks 2 days of age but was not present in the whole blood from the same chicks. Virus was not demonstrable in the brains or viscera of chicks of this series at 8 days of age, nor in the viscera at 11 days of age. The chicks hatched from "series D" eggs were bled from the hearts when 2 days old, but no virus was demonstrable in their blood. However, virus must have been present in the organs of the chicks at this time because it was later demonstrated there by calf inoculation in chicks of this series that were 5 days old. At 8 days of age, virus was no longer demonstrable in either the brains or the viscera of the "series D" chicks. The chicks hatched from "series E" eggs were discussed in the introduction and, as already indicated, had virus in a mixture of their brains and solid viscera when 3 days old.

It is apparent from the findings outlined in the table that persistence of rinderpest virus in the brains and solid viscera of chicks hatched from infected eggs is the rule. So far as can be told from the data we have accumulated, the virus persists until the chicks are at least 5 days old, but it disappears sometime before they reach 8 days of age. The failure to demonstrate virus in blood from 2 groups of chicks in which virus was present in the solid viscera suggests that the infection is not septicemic but rather that the virus

is in intimate association or relationship to fixed tissue cells in the organs or brain.

Most of the chicks hatched from infected eggs, and that were demonstrably carriers of rinderpest virus, appeared perfectly normal. A few were incoördinated and weak. Sections of brains from 2 of these incoördinated chicks failed to show any significant histopathologic lesions.

Two chicks from "series D" and 3 from "series C" were kept under observation for a month. They grew normally, were active and vigorous, and exhibited no detectable gross evidence to indicate that infection with rinderpest virus had affected them deleteriously in any way. Blood was obtained from these chicks when they were 1 month old and the pooled serums tested for antibodies against rinderpest virus. It was found that 1.0 cc. of the serum completely neutralized 100 m.i.d.'s of bovine virus as tested in a calf.

It is apparent that chicks hatching from eggs infected with rinderpest virus *via* the yolk sac remained infected with the virus until they were approximately 5 days old. Upon recovery from infection, the chicks developed specific virus-neutralizing antibodies. These observations suggested the possibility that chicks might prove susceptible to virus administered after hatching and might thus furnish us a small experimental animal for use in our work. To test the possibility, chicks were inoculated by various routes with rinderpest virus.

ATTEMPTS TO INFECT BABY CHICKS WITH RINDERPEST VIRUS

Chicks that were less than 1 day old at inoculation were used in most of our exprinments but older chicks also have been tried. Both bovine and egg-adapted viruses have been used. In the first attempt at infection, we tried to duplicate as nearly as possible the route used in infecting eggs, by injecting the inoculum into the unabsorbed yolk sac. In a day-old chick, the yolk sac is still quite large and can be injected by passing the needle through the abdominal wall just anterior to the stump of the invaginated yolk sac. One cubic centimeter of virus suspension was injected into the yolk sac through the abdominal wall in each of 12 chicks. Six days later, the chicks were killed and their brains and pooled solid viscera ground and suspended in physiologic saline. Six cubic centimeters of these suspensions failed to infect susceptible calves, indicating that virus had not been established in either the brains or solid viscera by this route of inoculation.

In other experiments, chicks were inoculated intraperitoneally, into the thorax, subcutaneously, intranasally while etherized, and by scarification. The chicks in these experiments were kept under observation for periods of either four or six days and were then killed. Ground suspensions of their solid viscera were tested for the presence of rinderpest virus by inoculation into susceptible calves. In no instance was virus demonstrated and it was concluded that chicks were not susceptible to infection by any of the routes tried.

Month-old chicks were fed large amounts of rinderpest virus mixed with their mash. Four days later they were killed and suspensions of their brains and organs tested for virus by inoculation into a susceptible calf. The calf remained normal, indicating that virus had not been established in the chicks by feeding.

The failure to establish rinderpest virus in baby chicks by any of the above routes of inoculation was a little surprising in view of the regularity with which chicks hatched from eggs inoculated during embryonation had been found to be infected. It would appear that factors other than the mere introduction of virus were requisite in establishing infection. It is probable that age is one of the factors involved and another may be the route by which the virus gains access to the chick. Virus inoculated into eggs in our routine manner reaches the embryo when it is but 7 days old and when the embryo is almost certainly in quite a different physiologic state than is the chick, even one but newly hatched. Furthermore, the virus on injection into the egg is not administered directly to the embryo but is placed in extra-embryonic tissues from where it can gain access to

the embryo by several routes. Perhaps if we could duplicate exactly this route we could succeed in infecting hatched chicks.

While we failed to infect chicks by any of the routes discussed above, infection can be achieved when the virus is administered intracerebrally. Cerebral infection, however, is a localized affair and is quite different from the more generalized infection encountered in chicks hatching from infected eggs. Instead of the virus being generally distributed, it is present only in the brains of cerebrally inoculated chicks and does not invade the solid viscera. Furthermore, although the brains of chicks inoculated intracerebrally with virus are infective for calves, four, six, or eight days later, only in 1 instance out of 4 attempts did we succeed in establishing virus in the brains of chicks of the second serial cerebral passage. In the remaining 3 series, second passage chick brains proved noninfective for susceptible calves. In the 1 series in which the second passage chick brains contained rinderpest virus, no virus could be demonstrated in the third serial passage chick brains. There is a strong probability in these experiments that little or no actual multiplication of virus occurred in the inoculated chicks but that the virus administered to them cerebrally merely persisted in the brains. Inability to transmit virus beyond the first cerebral chick passage in 3 cases and beyond the second passage in the fourth case supports this probability. However, if only survival is involved in these experiments, the virus persisted for an unusually long period of time; the brains of 1 series of chicks harvested six days, and of another series harvested eight days, after infection, brought calves down characteristically with rinderpest.

DISCUSSION

In the beginning, we had been particularly interested in the epidemiologic implications suggested by the initial observation that rinderpest virus survived in chicks hatched from infected eggs. We had toyed for awhile with the thought that perhaps rinderpest virus infection in chickens might be one, like choriomeningitis in mice or infectious anemia in horses, in which the virus persists for long periods of time in a relatively latent phase. If this were indeed the case, the chickens or, more probably, birds in general, might be looked upon as potential reservoir hosts in nature for rinderpest virus. Our subsequent experiments rather promptly demonstrated that the epidemiologic possibilities we had envisioned earlier were premature. In the first place, as it turned out, virus did not persist in chicks hatched from infected eggs beyond the fifth day and, even during the time the chicks were demonstrably infected, virus was not present in the blood stream where it would be potentially accessible to blood-sucking insects. Secondly, hatched chicks, even those only 1 day old, could not be infected with rinderpest virus by any route other than cerebral, and here actual multiplication of the virus was problematical. It became apparent, then, that so far as our information concerning chickens could be applied to birds in general, our observations had no epidemiologic applicability. They were of interest only as an instance, believed unique from the standpoint of current information, in which a virus cultivable in embryonating eggs and infective for the embryo, persists for a short while in the hatched chick.

SUMMARY

1) Chicks, hatched from eggs infected with rinderpest virus, contain the virus in their brains and in their solid viscera. The virus may persist in such chicks for as long as five days.

2) We were unsuccessful in infecting baby chicks with rinderpest virus administered by way of the unabsorbed yolk sac, intraperitoneally, intrathoracically, intranasally, subcutaneously, or by feeding or scarification.

3) Rinderpest virus administered intracerebrally to 1-day-old chicks is demonstrable in the brains of such birds for at least eight days. It is not transmissible in series directly from chick brain to chick brain.

Reference

Fourth Interim Report, Jan. 16, 1944.

Rinderpest

VII. The Attenuation of Rinderpest Virus for Cattle by Cultivation in Embryonating Eggs

CAPTAIN DUBOIS L. JENKINS, V.C., U.S. Army, and COMMANDER RICHARD E. SHOPE, M.C., U.S.N.R.

DURING the course of our studies dealing with the cultivation of rinderpest virus in embryonating eggs, we have observed a gradual attenuation of the egg-passage virus for calves. This has been noticeable not only in calves that have been permitted to run the entire course of their illness to either eventual death or survival, but also in animals that have been slaughtered at the supposed height of their disease on the fifth or sixth day after inoculation. In the latter group of animals, which constitute the large majority in our series, calves that have been infected with advanced passages of egg-propagated virus have seemed to show less abrupt febrile rises, their fevers have been less high, they have shown less early prostration, they have not exhibited such sudden and complete anorexia, and they have appeared generally less ill than have calves infected with virus of direct bovine origin. None of these differences, of course, constitute conclusive evidence for the attenuation of egg-grown rinderpest virus and they are mentioned only because they support evidence of an objective nature furnished by the group of animals observed throughout the entire course of their illness. It is largely with this latter group of experimental animals that the present report deals.

During the course of the present investigation, a total of 19 calves, which were normal so far as previous experience with rinderpest materials was concerned, have been infected with bovine-passage virus and observed throughout the course of their disease. Of these 19 calves, 3 eventually recovered while the remaining 16 died of rinderpest in from six to nine days after inoculation. The mortality rate for the bovine-passage virus, from which our egg-adapted virus was derived, is therefore something over 80 per cent. It constitutes a fairly regularly lethal infectious agent, and the occasional calf that escapes death from it does so only after a long and stormy course of illness. Transferred to growth in eggs, however, the virus gradually loses its capacity to kill and eventually reaches a stage in which it no longer makes calves seriously ill.

As recorded in an earlier report[1], rinderpest virus can be grown on the chorioallantoic membrane of the embryonating egg with regularity and ease, using infected bovine spleen as the original source of virus. In such cultures, virus is demonstrable in the chorioallantoic and amniotic membranes, but not elsewhere in the egg. In order to infect the embryos and to insure the presence of virus in the egg fluids, it is necessary to administer the virus to embryonating eggs by yolk sac inoculation. It was pointed out in a previous report that, though virus directly from infected calf spleen would infect the embryos and fluids of embryonating eggs when injected by way of the yolk sac, it could not be carried indefinitely in series in this way until it had been subjected to an adaptive series of passages in the chorioallantoic membrane[2].

The egg-cultivated virus included in the present report has the following history.

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The monthly reports occasionally referred to in this publication are official reports on the progress of the project, rendered monthly to the U. S.-Canadian Commission and filed in the Office of the Chief of Chemical Warfare. Interim Reports are special reports on important scientific accomplishments and developments in the project and rendered at such times as developments and circumstances warranted. Most of these Interim Reports constitute the basis of the present publication.

That recorded as having been grown on the chorioallantoic membrane was started originally from infected bovine spleen and has been transferred since then at three-day intervals on the membranes of 10-day embryonated eggs. The yolk sac series of cultures were initiated with 26th membrane-passage virus. They were transferred in 7-day embryonated eggs at intervals of either three or four days from the 27th to the 43rd serial egg passage. At the 43rd serial passage, the transfer interval was shortened to forty-eight hours and the series has been continued up to the present under this transfer routine.

The attenuation for calves that the virus has undergone during the course of its serial passage is shown in table 1.

The first test of the egg-cultivated virus for calf pathogenicity was made at the fourth chorioallantoic passage, and it was found that the virus was still capable of killing. However, even in this early passage, some evidence of attenuation was apparent. The 3 calves infected with the fourth membrane-passage virus survived for periods of ten, eleven, and twelve days, whereas, of the 16 fatal infections with bovine virus, as mentioned earlier, nine days had been the longest survival period. Ninth membrane-passage virus made calf 41 very sick, but the animal eventually recovered after an illness of fifteen days. Forty-third membrane - passage virus induced only a very mild illness in calf 238, manifested largely by five days of fever. Both of these calves on recovery were solidly immune to bovine spleen virus. Sixty-ninth membrane-passage virus, which we had anticipated would be more attenuated than that of the 43rd passage, induced an abrupt and very high fever (107 F.) in calf 337 to which it was administered. The animal was febrile for four days before it appeared definitely

TABLE 1—The Attenuation of Rinderpest Virus by Cultivation in Embryonating Eggs

Cultivation of the virus in eggs			Test for virulence in calves		Subsequent test for immunity to bovine virus	Interval between first infection and immunity test
Route of inoculation	Serial passage	Portion used for virus	Calf No.	Result		
Chorioallantoic membrane	4th	Chorioallantoic and amniotic membranes	31	Died—11 days		
Chorioallantoic membrane	4th	Chorioallantoic and amniotic membranes	32	Died—10 days		
Chorioallantoic membrane	4th	Chorioallantoic and amniotic membranes	27	Died—12 days		
Chorioallantoic membrane	9th	Chorioallantoic and amniotic membranes	41	Sick—15 days—recovered	Immune	21 days
Chorioallantoic membrane	43rd	Chorioallantoic and amniotic membranes	238	Mild illness—5 days—recovered	Immune	18 days
Chorioallantoic membrane	69th	Chorioallantoic and amniotic membranes	337	Sick—12 days recovered	Not tested	
Via yolk sac	31st	Allantoic and amniotic fluids	193	Sick—9 days recovered	Not tested	
Via yolk sac	41st	Hatched chicks (3 days old)	282	Mild illness—4 days—recovered	Immune	10 days
Via yolk sac	41st	Hatched chicks (6 days old)	298	Mild illness—6 days—recovered	Immune	19 days
Via yolk sac	48th	Allantoic and amniotic fluids	299	Mild illness—5 days—recovered	Immune	19 days
Via yolk sac	55th	Allantoic and amniotic fluids	334	Moderately ill—7 days—recovered	Not tested	
Via yolk sac	67th	Allantoic and amniotic fluids	332	No illness—2 temp. elevations	Immune	14 days
Via yolk sac	68th	Embryos	341	No illness—no temp. elevation	Immune	12 days
Via yolk sac	71st	Allantoic and amniotic fluids	343	No illness—1 temp. elevation	Immune	11 days

ill. It finally exhibited the characteristic, severe clinical picture of rinderpest and was febrile or ill for a total of twelve days. It eventually recovered after a stormy course.

The virus transferred by way of the yolk sac appears to have been more regularly and uniformly attenuated than that carried on the chorioallantoic membrane. Calf 193, infected with allantoic and amniotic fluids from 31st yolk sac-passage eggs, was ill for nine days and then recovered. This animal was the sickest of the group in which yolk-sac-passaged virus has been studied. Calves 282 and 298, infected with 41st yolk sac-passage virus in the organs, respectively, of 3-day and 6-day-old hatched chicks, were very mildly ill, though calf 282 underwent a febrile reaction of four days duration, and calf 298 underwent one of six days. Calf 282 was solidly immune to bovine spleen virus ten days, and calf 298, nineteen days, after their initial inoculation with yolk sac-passaged virus. Calf 299, infected with 48th yolk sac-passage virus in egg fluids, was only very mildly ill, though it was febrile for five days. It was solidly immune to bovine spleen virus nineteen days later. Calf 334 which received 55th passage virus was definitely ill and underwent a febrile reaction that persisted for seven days. The next 3 animals, calves 332, 341, and 343, infected respectively with 67th, 68th, and 71st yolk sac-passage virus were, clinically, not in the slightest ill. Calf 332 had a temperature of 104.4 F. on the afternoon of the fifth day, and 105 F. on the afternoon of the sixth day after inoculation but was otherwise afebrile. Calf 341 underwent no febrile reaction at all. Calf 343 had a temperature of 104.4 F. on the afternoon of the fourth day after inoculation but was otherwise afebrile. All 3 of these animals proved solidly immune to bovine spleen virus administered fourteen, twelve, and eleven days, respectively, after the initial inoculation with yolk sac-passage virus. It would seem from the findings that the virus transferred serially in embryonated eggs by way of the yolk sac had reached a point where it was almost completely innocuous for its natural host, yet still retained its ability to immunize against the fully virulent bovine-passage rinderpest virus.

Because of the possibility that virus attenuated by serial passage in eggs might prove usable as a live vaccine, it seemed of interest to try to learn whether its mild characters were fixed, or whether, in calves, it might revert to a more virulent form. Transmission by contact was first attempted, since it seemed possible that reversion might take place more readily under conditions of natural transfer of the virus. On the first day that calf 298 was febrile (*see* table 1), calf 307 was placed in the same pen. Likewise, on the first day of fever in calf 299, calf 311 was exposed to it. Contact was intimate. The normal animals ate from the same feed troughs, drank from the same buckets, and were closely confined in the same pens with the infected calves. In spite of this, neither of the exposed animals acquired rinderpest nor became immune to the virus. Calf 311 was kept in the same pen with calf 299 for sixteen days and then was inoculated with bovine spleen virus. It came down with characteristic rinderpest. In like manner, calf 307, kept in the same pen with calf 298 throughout, was still susceptible to rinderpest when challenged with virus after seventeen days' exposure. It was apparent that, so far as these two experiments told the story, the mild type of rinderpest caused by the egg-attenuated virus was not contagious in calves.

With the failure of the attenuated virus to transmit by contact, we next attempted to transfer it by means of defibrinated blood from an infected calf. This phase of our investigation is in a very preliminary stage and only a single experiment has, so far, been conducted. In this, blood was taken from the jugular vein of calf 343 (*see* table 1) on the fifth day after inoculation, which was the day after this animal had shown its single temperature elevation. Ten cubic centimeters of this blood, after defibrinating, was administered subcutaneously to calf 340. The calf remained, to all appearances, normal, though on the fifth day after inoculation it had a "high normal" temperature of 103.4 F. After eleven days of ob-

servation, the calf was given a dose of bovine spleen rinderpest virus that proved fatal for a control animal. Calf 340 was solidly immune and exhibited no clinical reaction whatsoever to the challenge infection. It was apparent from this experiment that the blood of calf 343 had, on the fifth day of its infection, contained rinderpest virus of the same mild, attenuated type as that present in advanced egg cultures, and that one calf passage did not cause it to revert to a more virulent form. Only further serial passages of this egg-adapted virus in calves will tell definitely whether or not it is actually "fixed."

As is indicated in the last two columns of table 1, calves which recovered from infection with attenuated egg-passage virus were immune to the fully virulent bovine-passage virus. This immunity was fully effective within ten days after administration of the egg virus, though most of the calves were not given their challenge dose of bovine virus until a somewhat longer period of time had elapsed. In order to learn whether attenuated virus would protect earlier, calf 342 was inoculated with 1.0 cc. of egg fluids of the 71st yolk sac-virus passage (the same virus inoculum used in the case of calf 343, table 1). On the morning of the fourth day after inoculation, the temperature of the calf rose to 104.6 F., evidently as a result of infection with the attenuated egg virus. At this time, ninety-six hours after administration of the egg virus, calf 342 was inoculated with approximately 2,000 m.i.d.'s of the fully virulent bovine-passage rinderpest virus. A normal animal, calf 339, was at the same time given a similar dose of bovine-passage virus to serve as a control. Calf 342 remained febrile for four consecutive days after receiving the bovine virus, but at no time appeared more than mildly ill, and exhibited diminution or loss of appetite for only three days. The control animal, calf 339, after an incubation period of two days, came down with characteristic rinderpest and died on the eighth day. At the time of the death of the control animal, calf 342 appeared perfectly normal, having made a complete, prompt, and uneventful recovery from its four days of mild illness. From this experiment, it would appear that attenuated egg-passage virus confers adequate protection against fully virulent bovine-passage rinderpest virus, even as early as four days after administration. This protection probably is not immunity in the usual sense, but partakes, rather, of the character of an interference phenomenon.

DISCUSSION

Prolonged passage of rinderpest virus in embryonating eggs has resulted in its definite attenuation for calves. This attenuation is more regular and uniform in virus transferred by yolk sac inoculation than it is in virus carried in the chorioallantoic membrane. Furthermore, in the yolk sac series, there is some evidence to suggest that rapidity of transfer through eggs plays a rôle in achieving attenuation. We gained the impression that the virus was less modified, from the standpoint of virulence, during the time that it was being transferred *via* the yolk sac at intervals of three or four days, than it was after we changed our routine and transferred it at intervals of forty-eight hours. Whether this impression has any basis in fact or not, the virus can be maintained perfectly satisfactorily, once it is fully adapted to transfer *via* the yolk sac, by serial passages at 48-hour intervals, and the time required to reach advanced passages is correspondingly shortened.

The illness induced in calves by the attenuated virus of advanced yolk sac passages is so mild as to practically escape recognition, unless one looks closely for it. Some transient elevation of temperature is the only regular clinical sign usually manifested. The mildness of the reaction and the regularity with which it is followed by firm immunity to fully virulent bovine-passage virus suggests the possibility that the egg-attenuated virus might be of use as a "live" vaccine in the event of an emergency. What evidence we have indicates that the disease caused by the egg-passage virus does not transmit by contact, nor does it revert to a more virulent form on a single serial calf passage. A more thorough and

complete study of this is indicated. Also, before the attenuated virus is seriously considered for field use, we should have information of a more definite character as to whether or not it is actually "fixed", or whether, on further back passages through cattle, it will revert to its original fully virulent form. If it is "fixed", it would seem to constitute a relatively safe agent to use even in the state of attenuation it has now reached in the yolk sac-passage series. More prolonged serial passage by this route probably will attenuate it still further, but it may also diminish its capacity to immunize as well. Certainly, however, before it is contemplated for any large scale use, it should be given a test in a large series of cattle under actual field conditions. We have studied it only under the somewhat idealized conditions of the laboratory and its behavior under more rugged field conditions might alter our views as to its safety.

The virus that has been transferred only by way of the chorioallantoic membrane, even though it has been subjected to as many serial passages in eggs as the yolk sac-transmitted virus, would be quite unsafe to use as a "live" vaccine. The disease it produced in its 69th passage in calf 337 was of far too serious a character clinically to constitute a practical means of inducing immunity to rinderpest. Under field conditions, calf 337 would have almost certainly died. There is, of course, the possibility that this calf may have been an abnormally susceptible animal and that, had it received yolk sac-passage virus instead of the membrane-passage virus, it would still have sickened in the same manner. Study of the behavior of the attenuated virus in a much larger series of animals is indicated in order to properly adjudge its potential possibilities, both from the standpoint of hazards involved in its use and benefits to be derived in the form of immunity.

The speed with which the attenuated yolk sac-passage virus assures protection against rinderpest recommends it highly for consideration as a vaccine for use in the field during an actual epizoötic. The protection afforded is apparently effective within four days by virtue of "interference" and, after this, true immunity is established. It seems likely that some degree of protection would be afforded almost as soon as the attenuated virus was administered. Thus, in an actual outbreak of rinderpest in which virulent virus was spreading actively and rapidly, the generous use of "live" attenuated virus would save many animals, by virtue of its rapid effectiveness, that would be lost if they received only the more slowly immunizing, inactivated bovine tissue vaccine. Attenuated virus could be prepared in tremendous quantities on very short notice.

SUMMARY

1) Prolonged serial passage of rinderpest virus in embryonating eggs attenuates it for calves.

2) The attenuation appears to be more regular and uniform when the virus is transferred by way of the yolk sac than when it is carried in the chorioallantoic membrane.

3) The attenuated virus solidly immunizes calves against fully virulent bovine spleen virus. This immunity is effective within ten days.

4) The mild rinderpest caused by egg-attenuated virus does not appear to be contagious.

5) Attenuated virus apparently "interferes" with virulent bovine spleen virus, and a calf that received the attenuated agent only four days before the virulent one was protected against the severe and lethal effects of the latter.

6) If further investigation shows the attenuated virus to be as mild and harmless for calves as preliminary experiments indicate it to be, its use as a "live" vaccine in the field during an emergency is suggested.

7) "Live" attenuated virus vaccine would give adequate protection during an epizoötic more rapidly than would inactivated bovine tissue vaccine.

References

[1]First Interim Report, July 16, 1943.
[2]Fourth Interim Report, Jan. 16, 1944.

Rinderpest

VIII. Rinderpest Infection in Rabbits

CAPTAIN JAMES A. BAKER, V.C., U.S. Army

THE ABILITY of the virus of rinderpest to produce illness in rabbits has been a controversial topic for many years. Hall[1] investigated the problem and reported failure in his efforts to transmit the disease by intravenous inoculations of infective blood. He was unsuccessful in effecting signs that could be considered reliable, and blood from inoculated rabbits failed to infect calves. A different result was reported by Nakamura, *et al.*[2, 3, 4, 5], who transferred the infection to rabbits. They showed a febrile reaction and this effect was neutralized by immune serum.

Using the strain of virus available in this laboratory, it was impossible with the methods described by others to transfer rinderpest virus in rabbits beyond one passage. It was only by using a new method that serial passage was accomplished and definite signs of illness produced. The technique employed and the results obtained are reported herewith.

Transmission of Rinderpest to Rabbits.—Although most of the experimental work reported in the literature referable to the transmission of rinderpest from calves to rabbits mentions that the inoculations were given intravenously, this route, as well as injections subcutaneously and intraperitoneally, was used. The material employed for the original inoculations into rabbits was spleen from a calf made ill by a subcutaneous injection of virus that had been maintained in calves only. A portion of spleen was weighed and a 10 per cent suspension in physiologic saline solution made by use of a glass grinder. The suspension was allowed to stand a few minutes to permit settling of coarse particles and the supernatant used for inoculation.

White rabbits of the New Zealand breed were used as experimental animals. They were apparently healthy and their temperatures ranged from 102.2 to 103.5 F. when kept under experimental conditions. In no instance was a temperature higher than 104.0 F. recorded for normal control animals. Rabbits from 3 to 5 months old were inoculated intravenously, intraperitoneally, and subcutaneously with 1.0 cc. of the tissue suspensions. Thereafter, temperatures were taken twice daily. Animals used for serial passages were killed three to five days after inoculation, and suspensions of their spleens, prepared in the same way as the calf material, were inoculated into rabbits and calves. The results are presented in table 1.

TABLE 1—Transference of Rinderpest Virus From Calves to Rabbits

Route of inoculation	Rabbit No.	Passage No.	Signs of illness	Test for virus in spleens of rabbits by inoculating calves
Subcutaneous	3	1	None	Not present
Intraperitoneal	13	1	None	Not present
	14	2	None	Not present
	48	1	None	Not present
Intravenous	16	1	None	Present
	20	2	None	Not present
	49	1	None	Present

As determined by tests in calves, virus was present in the spleens of rabbits inoculated intravenously with material from calves, but not in those given the virus subcutaneously or intraperitoneally. The virus was not passed serially in rabbits by any route from these inoculations.

Establishment of the Virus in Serial Passages.—As shown in table 1, spleens from rabbits inoculated intravenously contained virus and, when inoculated into a calf, caused illness. From this calf, a second series (B) of rabbit passages was initiated by the same method used for the original inoculation. After two passages, a calf was

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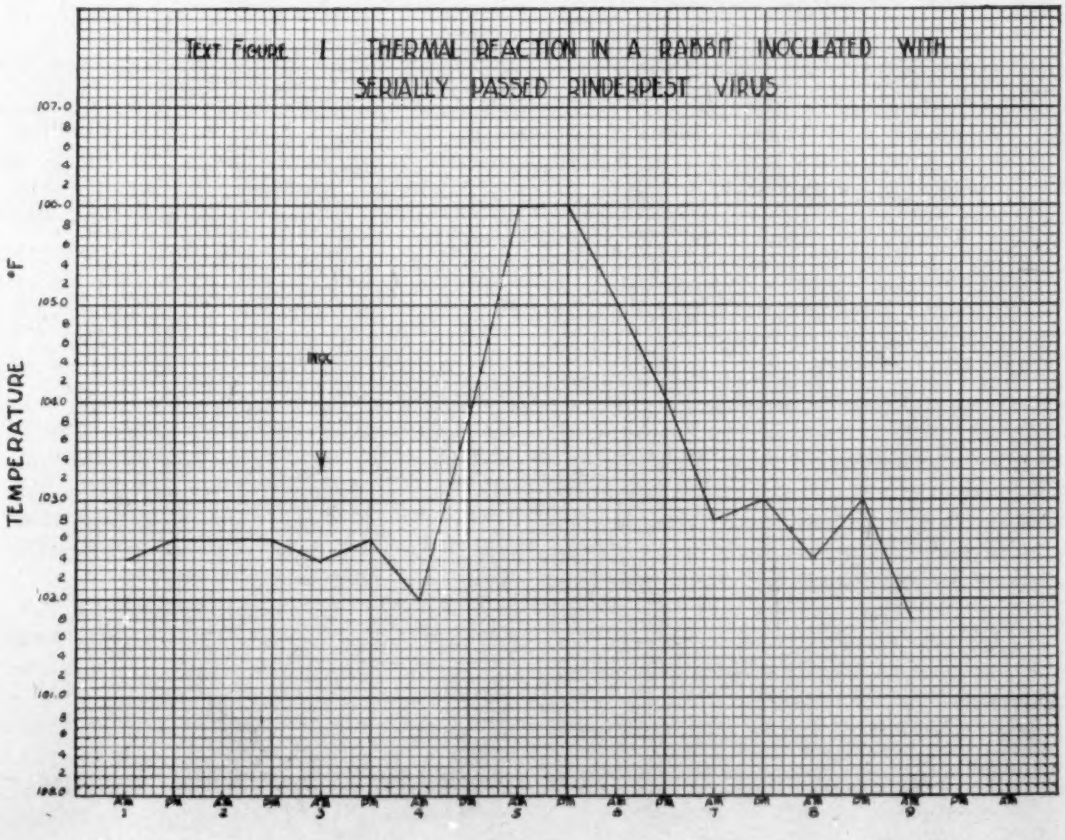
inoculated with a suspension of spleen from these rabbits, and a third series (C) was started from this animal. This series, therefore, represented virus that had been in two alternating passages between calves and rabbits.

Spleens from certain rabbits and from calves in the different series (A, B, and C) were titrated for virus content by inoculation into calves. These results are shown in table 2 which, for purpose of clarity, shows only some of the calves and rabbits used in these experiments.

It can be seen in table 2 that rinderpest virus was established in rabbits in a direct transmission from calves (Series A-1) and that it produced no signs of infection. In a second passage (Series A-2), the virus was lost, for a suspension of spleen from this rabbit produced no illness in calves. Signs of disease for the first time were noted in rabbits in the passage (Series B-1) that had alternated once between calves and rabbits. Again, after three passages (Series B-3), the virus was lost, although it was shown to be present in the second passage. It was only after a further series (C) that the pathogenicity of the virus of rinderpest was increased to the extent that it produced definite signs of disease which were regularly reproduced through five serial passages. This experiment was repeated, and again the results were the same.

It was found in the titration of virus in the spleens from rabbits in the different series that the amount of virus became greater as the pathogenicity increased. When the spleen contained only ten m.i.d.'s per gram of tissue for calves, it no longer caused infection in a further passage, and ten times that amount was used to maintain the virus in serial passage. The spleens from calves inoculated with rabbit material contained at least 1,000 m.i.d.'s.

Signs of Illness.—Rabbits inoculated with the virus that had been passed in alternat-



ing series between calves and rabbits showed a sharp rise in temperature thirty-six to forty-eight hours after inoculation. A typical febrile reaction, as shown in text figure 1, generally persisted for about two days and then returned to normal. At the height of the fever, the respirations were markedly increased, the animal refused food and exhibited signs of malaise. Occasionally, diarrhea was observed. Prompt recovery followed the return of a normal temperature.

Pathology.—Animals subjected to autopsy while signs of the infection were present showed a reddening of the mucosa of the stomach and intestines. The mucosa of the stomach in the glandular region was covered with a thin, whitish exudate that occasionally formed a membrane which could be detached intact from large areas. The spleen usually showed enlargement. No pneumonia or other lesions were noted in any organs.

Immune Studies.—A 10 per cent suspension in physiologic saline solution, of spleen containing virus that produced definite signs of illness in rabbits, was centrifuged at 1,500 r.p.m. for five minutes. Portions of the supernatant were removed and mixed with equal amounts of (a) normal bovine serum, (b) serum from a calf convalescent from rinderpest, and (c) serum obtained from a calf thirteen days after it had received an immunizing injection of chloroformized bovine tissue vaccine. The mixtures were allowed to stand in the ice-box for two hours and 2.0 cc. were inoculated intravenously into rabbits. Daily tempera-

TABLE 2—Rinderpest Virus Established in the Rabbit from Calves by Alternating the Serial Passages between the Species

Calf Series	Rabbit inoculations: Serial passage	Animal No.	Thermal reaction (peak temp.-F.)		Production of disease in calves with spleen suspensions from rabbits: Dilution	Calf No.	Result
A (from calf 1)	1	16	No fever	102.8	10^{-1}	2	Rinderpest infection
					10^{-2}	3	No rinderpest
	2	20	No fever	103.5	10^{-1}	4	No rinderpest
B (from calf 2)	1	19	Fever	106.4	10^{-2}	5	Rinderpest infection
					10^{-3}	6	No rinderpest
	2	21	Fever	104.2	10^{-1}	7	Rinderpest infection
					10^{-2}	8	No rinderpest
	3	25	No fever	103.4	10^{-1}	9	No rinderpest
C (from calf 7)	1	26	Fever	107.2			
	2	28	Fever	106.2			
	3	32	Fever	106.2	10^{-3}	10	Rinderpest infection
					10^{-4}	11	No rinderpest
	4	33	Fever	106.4			

TABLE 3—Cross-Neutralization Tests in Calves and Rabbits

Virus	Serum	Animal used in Test	Result: Thermal Reaction	Result: Peak (F.)	Reinoculation Result: Thermal Reaction	Reinoculation Result: Peak (F.)
Rabbit—passaged	Normal bovine Calf 12	Rabbit 30	Fever	106.8	No fever	103.6
Rabbit—passaged	Convalescent bovine Calf 12	Rabbit 29	No fever	102.8	Fever	106.0
Rabbit—passaged	Prevaccination bovine—Calf 14	Rabbit 34	Fever	106.6	No fever	102.8
Rabbit—passaged	Postvaccination bovine—Calf 14	Rabbit 35	No fever	103.8	Fever	104.6
Bovine—passaged	Normal rabbit 11 and 12	Calf 262	Fever	107.8	Not tested	
Bovine—passaged	Immune rabbit 11 and 12	Calf 274	No fever	103.2	Fever	106.8

tures were taken over a period of fourteen days and these rabbits were then reinoculated with pathogenic virus. Neutralization tests, prepared as for rabbits, were done in calves with serums from normal rabbits and from recovered rabbits. The virus used had been passaged only in calves. These results are shown in table 3.

As shown in table 3, immune bovine serums neutralized rinderpest virus pathogenic for rabbits, and immune serums from rabbits likewise neutralized the virus that had been passaged only in calves. Complete neutralization was shown, in that the animals receiving the mixture of virus and immune serums became ill when reinoculated fourteen days after the test inoculation; whereas, those that had been given the mixture with normal serums and had developed illness were immune.

DISCUSSION

The evidence permits the conclusion that rinderpest virus can be established and maintained in serial passage in rabbits by intravenous inoculation, but not when given intraperitoneally or subcutaneously. Certainly the cross-neutralization tests offer conclusive proof that the signs of illness were produced by the virus of rinderpest and not by another infection encountered in passage. However, it is only after a series of alternating passages with calves that the pathogenicity can be increased to the extent that definite signs of disease are noted. As demonstrated in the text, it would be impossible to establish and maintain the virus in serial passage in rabbits without employing this alternating method of inoculation. Since it is a new technique not previously reported, it seemed worthwhile to discover the mechanism of its success.

Consideration of the rinderpest virus-rabbit relationship showed that although 10,000 m.i.d.'s for calves per gram of tissue were given rabbits in the original inoculation, only ten m.i.d.'s were produced in their spleens. Since 100 m.i.d.'s are required to establish the virus in rabbits, this amount was not sufficient to overcome the threshold of infection and so the serial passage ended. However, the virus content was restored to more than 1,000 m.i.d.'s in inoculated calves. The success of the method consisted, therefore, in alternating passages between calves and rabbits in order to keep the virus content above the threshold of infection for rabbits. The virus eventually gained sufficient pathogenicity so that it produced this amount in the spleens of inoculated rabbits and the serial passage was maintained.

This procedure should be found useful for many viruses.

SUMMARY

1) Rinderpest virus was transferred from calves to rabbits with suspensions of infective spleen given intravenously, but it could not be maintained in a further passage. It was only by alternating the inoculations between calves and rabbits that the virus became pathogenic for rabbits.

2) This method to increase the pathogenicity of rinderpest virus for a resistant animal would seem applicable to other viruses and an explanation was sought for its success. This is discussed in the text.

3) Cross-neutralization tests, using immune serums from calves and rabbits, showed that the illness produced was caused by rinderpest virus and not by another agent encountered in passage.

4) Contrary to some reports in the literature, and in agreement with others, it is concluded that rinderpest virus can be made to produce definite signs of illness in rabbits.

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Rinderpest

IX. Neutralization Tests in Rabbits as a Measure of the Immune Responses in Calves to Vaccination against Rinderpest

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SERUMS of cattle recovered from rinderpest neutralized the virus so that it no longer produced illness when given to susceptible animals.[1] For many years, the only animals available for neutralization tests were cattle. Recently,[2] a method was reported by which the virus of rinderpest regularly can be made to produce a marked thermal reaction in rabbits, and it was shown that this sign was repressed when serum from recovered calves was added to the inoculum. Since it is possible, therefore, to do a neutralization test in rabbits, it seemed worthwhile to examine the neutralizing capacity of serum from vaccinated calves. Two questions immediately posed were: Is there any correlation between the immunity produced by vaccination and the development of neutralizing antibodies; and, if so, is there any relationship between the vaccine given and the immune response? In seeking the answer to these questions, certain data were obtained which are presented in this report.

THE NEUTRALIZATION TEST

Most of the rabbits used throughout these tests were purchased locally, but some were reared at this station. Various breeds, ages, sizes, and sexes were represented in the experiments. Prior to inoculation, several temperatures were taken to determine normalcy. As can be seen in text figure 1, these temperatures usually ranged from 102 to 103 F., although 101 F. and 104 F. were recorded as extremes. Only slight differences were noted between individuals, and the younger and smaller rabbits were less useful because of unstable temperatures. All individuals reacted similarly when given virus.

Spleens from calves served as the source of virus, thus providing a large amount of material for inoculation purposes. Virus that was pathogenic for rabbits was inoculated into calves subcutaneously and after two or three high temperatures were recorded, they were killed and their spleens removed and kept in the refrigerator at —6 F. In all, spleens from 3 calves were used for test purposes.

A portion of spleen from a calf was removed, weighed, and a 10 per cent suspension in physiologic saline (10^{-1} dilution) made by use of a glass grinder. The suspension was centrifuged at 1,500 r.p.m. for five minutes, the supernatant was poured off, and tenfold dilutions of it were prepared in saline. Rabbits were inoculated intravenously with 1 cc. of these dilutions or 2 cc. if mixed with an equal amount of normal bovine serum. The results are shown in text figure 1, where it can be seen that both the 10^{-1} and 10^{-2} dilutions regularly produced a high thermal reaction with little spread around the mean temperature. The 10^{-3} dilution caused a definite thermal reaction in most rabbits, but some showed temperatures in the normal range. All animals receiving each dilution were reinoculated with a 10^{-1} dilution of the virus ten days after the initial injection. Those animals receiving the 10^{-1} and 10^{-2} dilutions and most of them that had been

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The monthly reports occasionally referred to in this publication are official reports on the progress of the project, rendered monthly to the U. S.-Canadian Commission and filed in the Office of the Chief of Chemical Warfare. Interim Reports are special reports on important scientific accomplishments and developments in the project and rendered at such times as developments and circumstances warranted. Most of these Interim Reports constitute the basis of the present publication.

given the 10^{-3} dilution showed no temperature rise following the second inoculation. However, the peak temperature in these animals that showed a thermal response was considerably lower than the control animals. There was not complete correlation between the thermal rise after the primary inoculation and temperature increase following the second one in the rabbits given the 10^{-3} dilution. For example, an occasional animal maintained a normal temperature throughout, while others had a peak temperature of 104.4 F. following each inoculation. The 10^{-4} dilution produced no thermal reaction and no immunity was demonstrated. It was considered, therefore, that the 10^{-3} dilution represented a number of minimal infective doses (m.i.d.'s) of virus between 1 and 10, and the other dilutions were 10 to 100 (10^{-2}) and 100 to 1,000 (10^{-1}) m.i.d.'s. At the end of a series of tests, each spleen was again titrated for virus content. Any decrease in virus titer of a spleen invalidated the tests done with it; this only occurred in one instance.

Similar suspensions and dilutions were prepared as above and 1.5 cc. of each was mixed with an equal portion of test serum. After an incubation period of two hours in the refrigerator, 2 cc. of each mixture were given intravenously to a rabbit. Rectal temperature readings of the animals were made twice, daily. Rabbits that had a temperature of 104 F. or lower were considered to have received a neutral mixture, while those showing 104 to 104.5 F., a doubtful reaction, and 104.5 F. or higher indicated no neutralization.

The test serums came from calves at the time of vaccination and again two weeks later, *i.e.*, just prior to the determination of immunity by the inoculation of pathogenic virus. These animals were given either inactivated bovine-tissue vaccine[3] or attenuated chicken embryo virus vaccine.[4,5] Other serums, studied for comparative pur-

Text Figure 1 THERMAL REACTION CURVES OF RABBITS GIVEN VARIOUS DILUTIONS OF VIRUS WHOSE PEAK TEMPERATURE APPROXIMATES THE MEAN

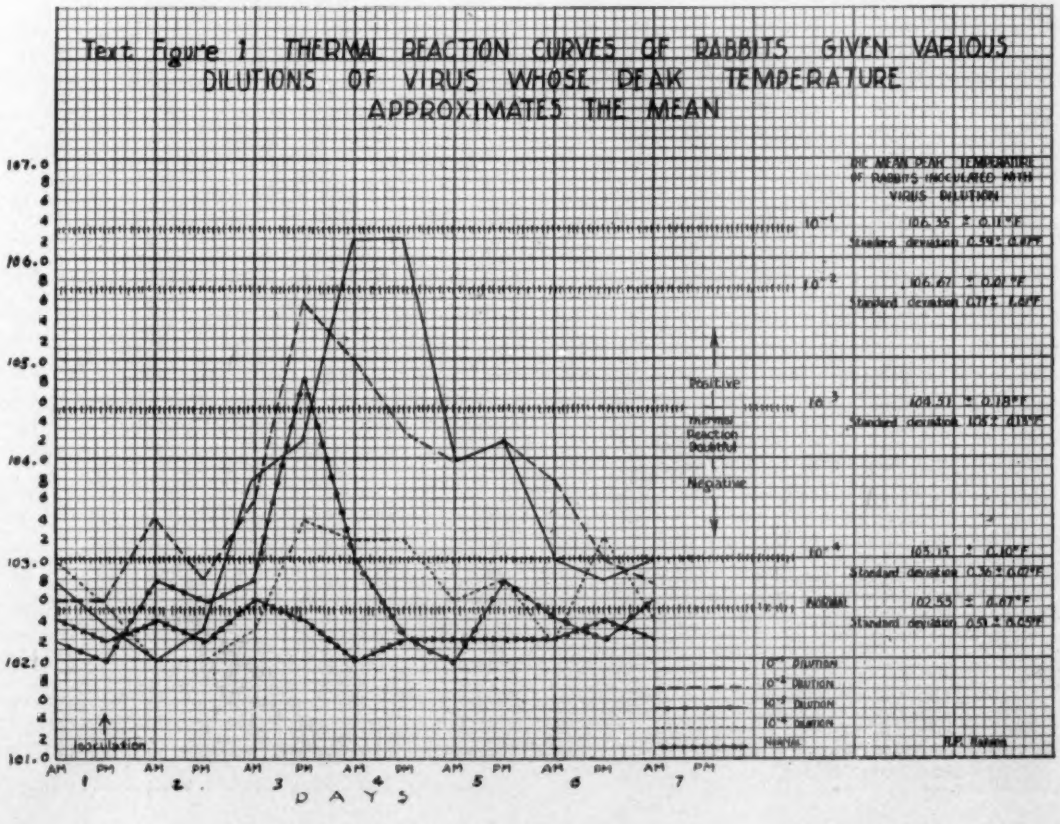

TABLE I—Results of Neutralization Tests in Rabbits with Serums from Calves Vaccinated with Inactivated Bovine Tissue Vaccines

Calf No.	Given vaccine				Response to test dose of virus	Serum status	Rabbit Neutralization test results	
	No.	Age tissue before preparation (days)	Age of vaccine after preparation (days)	Amount (cc.)			10-100 m.i.d.'s	100-1,000 m.i.d.'s
321	Batch 23	4	99	10	Non-immune	Pre-vac	—	
						Post-vac	+	—
322	Batch 42	172	6	10	Non immune	Pre-vac	—	
						Post-vac	—	
323	Batch 41	100	6	10	Immune	Pre-vac	—	
						Post-vac	+	—
324	Batch 40	66	6	10	Immune	Pre-vac	—	
						Post-vac	+	—
325	Batch 39	1	6	10	Immune	Pre-vac	—	
						Post-vac	+	—
326	Batch 38	2	31	10	Non-immune	Pre-vac	—	
						Post-vac	+	—
327	Batch 37	2	33	10	Immune	Pre-vac	—	
						Post-vac	+	—
328	Lot 9	142	50	20	Immune	Pre-vac	—	
						Post-vac	—	
329	Lot 8	2	110	20	Non-immune	Pre-vac	—	
						Post-vac	—	
330	Lot 7	105	93	20	Immune	Pre-vac	—	
						Post-vac	—	
353	Lot 1	13	205	10	Non-immune	Pre-vac	—	
						Post-vac	—	
366	Lot 1	13	260	20	Immune	Pre-vac	—	
						Post-vac	+	—
369	Batch 44	195	40	20	Non-immune	Pre-vac	—	—
						Post-vac	—	—
372	Batch 46	0	11	5	Immune	Pre-vac	—	—
						Post-vac	—	—
373	Batch 46	0	11	10	Immune	Pre-vac	—	
						Post-vac	+	—
400	Lot 4	39	265	20	Non-immune	Pre-vac	—	
						Post-vac	—	
403	Lot 11	40	102	20	Immune	Pre-vac	—	
						Post-vac	+	+
404	Batch 50	250	9	20	Non-immune	Pre-vac	—	
						Post-vac	—	
405	Batch 48	206	11	20	Immune	Pre-vac	—	
						Post-vac	—	
407	Batch 59	97	5	20	Immune	Pre-vac	—	
						Post-vac	+	—
411	Batch 49, 51, 53, 56	9	9	10	Non-immune	Pre-vac	—	
						Post-vac	—	
413	Batch 46	0	37	10	Immune	Pre-vac	—	
						Post-vac	+	—
506	Lot 12	0	126	20	Non-immune	Pre-vac	—	
						Post-vac	—	
526	Lot 15	82	29	20	Immune	Pre-vac	—	
						Post-vac	+	—
528	Lot 17	8	22	10	Immune	Pre-vac	—	
						Post-vac	+	—

+ Indicates neutralization.
— Indicates no neutralization.

poses, came from animals that had recovered from experimentally produced rinderpest. In tables 1 and 2 are shown the individual calves, the amount of vaccine given, its history and immunizing properties, as well as the results of the neutralization test. A general summary of the immune response in calves to each type of vaccine, as measured by the amount of neutralizing antibodies present in their serums, is given in table 3. The prevaccination serums and serums of animals recovered from rinderpest are included.

100 days while the other was given in a low dosage, *i.e.*, 5 cc.

For those calves classified as nonimmune to the challenge dose of virus after receiving the inactivated bovine tissue vaccine, the serums of 2 neutralized 10 to 100 m.i.d.'s. The protocols of these animals reveal that four and five days after test virus was given, they showed a slight febrile reaction of short duration. No other signs of illness were noted. When killed and examined one day after the control animal died of typical rinderpest, the only

TABLE 2—Results of Neutralization Tests in Rabbits with Serums from Calves Vaccinated with Lyophilized Attenuated Virus from Embryonated Eggs

Calf No.	Vaccine No.	Response to test dose of virus	Serum status	Neutralization test results	
				10-100 m.i.d.'s	100-1,000 m.i.d.'s
481	C 5	Immune	Pre-vac	—	Not tested
			Post-vac	—	Not tested
482	C 4	Immune	Pre-vac	—	Not tested
			Post-vac	+	+
498	C 7	Immune	Pre-vac	Not tested	Not tested
			Post-vac	Not tested	+
561	C 8	Immune	Pre-vac	Not tested	Not tested
			Post-vac	Not tested	+
514	C 4	Immune	Pre-vac	—	Not tested
			Post-vac	+	—
517	C 9	Immune	Pre-vac	Not tested	Not tested
			Post-vac	+	—
518	C 10	Immune	Pre-vac	Not tested	Not tested
			Post-vac	Not tested	+
519	C 11	Immune	Pre-vac	Not tested	Not tested
			Post-vac	Not tested	+
520	C 12	Non-immune	Pre-vac	Not tested	Not tested
			Post-vac	—	Not tested
521	C 13	Non-immune	Pre-vac	Not tested	Not tested
			Post-vac	—	Not tested

+Indicates neutralization.
—Indicates no neutralization.

In the summary of results shown in table 3, it can be seen that all calves given the inactivated bovine tissue vaccine, and later shown to be immune to the test dose of virus, developed a neutralizing capacity for 10 to 100 m.i.d.'s except 5 animals. Of these, the serum of 1 animal neutralized 100 to 1,000 m.i.d.'s, whereas no neutralization was demonstrated for the other 4 animals. A study of table 1 showed that three of those vaccines which did not produce neutralizing antibodies were prepared from tissue stored at —6 F for more than

lesion found was a pneumonia. The vaccines given these animals have a history similar to those that immunized. The other 8 nonimmune calves, when given the test dose of virus, showed a fever curve and autopsy findings resembling the experimental disease. Their serums did not neutralize. An examination of the history of the vaccines given these animals showed that three were prepared from tissues stored for longer than 100 days. Another of these vaccines, held for over 200 days after preparation, was then given in a 10-cc. dose.

It later immunized a calf when 20 cc. were given. One other vaccine was made from tissues of calves that had been inoculated with virus passaged in rabbits instead of the usual bovine-passaged virus. The remaining three vaccines, according to their history, did not differ from those that produced immunity.

It is shown in table 2 that immunity against a test dose of virus was found in 8 calves vaccinated with attenuated chicken embryo virus vaccine. The serums from 5 of these animals neutralized 100 to 1,000 m.i.d.'s, 2 neutralized 10 to 100 m.i.d.'s, and 1 lacked this capacity. No immunity or neutralizing antibodies were produced by two of the vaccines.

The serums of the 5 calves recovered from the disease all possessed a neutralizing capacity for 100 to 1,000 m.i.d.'s. None of the serums taken from uninoculated calves neutralized (table 3).

TABLE 3—A Summary of Vaccination Results in Calves Against Rinderpest as Determined by Neutralization Tests in Rabbits (Prevaccination Serums and Serums of Calves Recovered from Rinderpest Are Included)

Inoculation	Calf response to test dose of virus	Number of calf serums tested	Neutralizing effect of serum		
			None	10-100 m.i.d.'s	100-1,0000 m.i.d.'s
Inactivated bovine tissue vaccine	Immune	15	4	10	1
	Non-immune	10	8	2	
Attenuated egg virus vaccine	Immune	8	1	2	5
	Non-immune	2	2		
None		25	25		
Virus	Immune	5			5

DISCUSSION

It was found that the rabbit is useful to show the neutralizing ability of serums from cattle immune to rinderpest. Unfortunately, the size and care of rabbits prohibits their use in large numbers. It is for these reasons that 50 per cent end point titrations were not made and dependence was placed on the semiquantitative method of testing a range of minimal infective doses. If sufficient numbers of rabbits had been used to determine a 50 per cent end point, probably the sensitivity of each test would have been increased and smaller amounts of neutralizing antibody detected. However, it is doubtful if the end would justify the means and, from the practical standpoint, the method reported above was found to be satisfactory.

In answer to one of the questions mentioned in the introduction, there seems to be a definite correlation between the immunity produced by vaccination and the development of neutralizing antibodies, and this is particularly marked if we reconsider the 2 calves which showed neutralizing antibodies in their serums but were classified as nonimmune. As stated in the results, these animals showed signs of a mild febrile reaction only, and the autopsy findings were not typical of rinderpest. It might be more logical to include them with the immune calves for, even if the elevation in temperature was due to the inoculation of virus, a partially immune state existed. Furthermore, an intercurrent infection that might account for the fever was not eliminated from the picture. Including these 2 calves, there are 25 animals that showed immunity following vaccination. Of these, serums from 20 were found to neutralize the virus in rabbit tests. The history of three of the five vaccines that did not produce a neutralizing capacity for 10 to 100 m.i.d.'s caused doubt as to their ability to produce a good immunity. The age of the tissue used for their preparation was more than 100 days, and three other vaccines prepared in a similar manner did not immunize calves to rinderpest. Still another of these five vaccines was given in a 5-cc. dose, an amount which should be

at the lower limit of dosage, since many other vaccines similarly prepared and tested failed to produce immunity. Therefore, the production of few if any neutralizing antibodies could be expected from these four vaccines, and any attempt to demonstrate neutralizing capacity should be in tests against small amounts of virus by the 50 per cent end point method. There remains one vaccine, an attenuated chicken embryo virus one, which immunized and should have produced a high neutralizing antibody content, but failed to do so. Assuming these interpretations to be correct and, not counting the three poor vaccines nor the one given in low dosage, there is a correlation in 20 instances (95.2 per cent) and none for 1 calf (4.8 per cent).

It is interesting to note that none of the serums taken from uninoculated calves showed any capacity to neutralize, while those from animals recovered from rinderpest neutralized the largest amount of virus used in the tests.

The other question cannot be given a definite answer because the number of serums tested is not large enough. The results merely indicate that the attenuated chicken embryo virus vaccine usually produced a higher neutralizing antibody content than the vaccines made from bovine tissue virus.

From the evidence produced, it is concluded that neutralization tests in rabbits give a good indication of whether immunity is produced in calves either by vaccination or previous infection. Since the virus of rinderpest supposedly produces a septicemia and sets up infection through the blood stream, it appears a good assumption that neutralizing antibodies would constitute an effective barrier to infection, or at least indicate a good defense against it. Therefore, the neutralization test in the rabbit appears to be one practical method of determining whether immunity is produced by a vaccine to the extent that good vaccines produce neutralizing capacities in 95.2 per cent of the calves vaccinated. Hence, a vaccine that failed to produce neutralizing antibodies for 10 to 100 m.i.d.'s when given to a calf, even though this animal proved immune to a test dose of virus, should be tested again, and a vaccine that did produce neutralizing antibodies should be considered good whether or not the test animal showed a mild febrile reaction when challenged with virus. Of course, if a test animal developed typical rinderpest when given the test dose of virus and had neutralizing antibodies in its serum, the vaccine should be discarded. An animal of this type has not been found in our tests. Also, it is thought to be a good procedure to collect appropriate serums from animals vaccinated in the field and test them as a check of the procedure. Again, the demonstration of neutralizing antibodies in representative samples would mean effective vaccination.

SUMMARY

A method is outlined of doing neutralization tests in rabbits that measures the antibody content in serums from animals, either vaccinated against or recovered from experimental rinderpest. The practical significance for determining the efficiency of vaccination against rinderpest is discussed.

Of 65 serums tested, 32 were from calves that did not show signs of rinderpest when given a test dose of virus. Of these, 27 possessed neutralizing capacity while five had none. The discussion in the text shows that 4 of these calves were given poor vaccines that were not expected to produce a good immunity. In only 1 there was no correlation between immunity and the development of neutralizing antibodies. The remaining 33 of 65 serums were taken from uninoculated calves or animals that did not develop immunity after vaccination. None of these serums neutralized the virus. It is concluded that there is a definite correlation between immunity and the development of neutralizing antibodies.

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Rinderpest

X. The Response of Guinea Pigs to the Virus of Rinderpest

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EXPERIMENTAL WORK with any disease is facilitated by establishing the infection in small laboratory animals. In addition to practical benefits, useful information about the biologic properties of the agent under investigation is obtained. For example, after establishing the virus of rinderpest in rabbits, a number of problems were capable of immediate investigation that otherwise would have been extremely difficult to study in calves.

Undoubtedly, the range of our investigations would be extended further if several laboratory animals could be infected. An obvious study would be to determine the effect of rinderpest virus, particularly the avianized form, in guinea pigs, with emphasis upon observation for signs of illness, production of active immunity and the development of neutralizing antibodies. Positive results would provide a test animal that would be a substitute for calves used for potency tests of egg vaccines. Although the guinea pig is reported to be completely insusceptible to the virus of rinderpest, it appeared worthwhile to investigate the possibility of their use by applying the method employed with rabbits.[1]

EXPERIMENTAL

Inoculation Studies.—Since signs of infection were produced in rabbits by alternating the passages between them and calves, the same method was followed in studying the effects of rinderpest virus in guinea pigs. Two forms of the virus were used: a bovine-passaged strain and the same one that had been made pathogenic for rabbits. The lapinized form was employed because it produced fever in the rabbit and, hence, was considered "tagged." It, therefore, could be followed in the study by the signs produced in rabbits.

Portions of spleen were removed from a calf infected with virus, weighed, and a 10 per cent suspension made in physiologic saline by use of a glass grinder. This suspension was then centrifuged for five minutes at 1,200 r.p.m. and the supernatant used for inoculation. Groups of 2 or 3 guinea pigs each were given intravenously 1 cc. of the suspension. Temperatures were taken twice daily and then 1 or 2 of the animals were killed three to four days after inoculation, and their spleens removed. Suspensions of these spleens were prepared as above and similarly inoculated into other guinea pigs for further passage. Also, various tenfold dilutions were given subcutaneously to calves. Alternating passages from calves showing signs of rinderpest to guinea pigs were done, as for the original passage. These results are shown in table 1. In addition, 1 cc. of 10 per cent suspensions, in physiologic saline, of spleens from calves at each step of the alternating passage of "tagged" lapinized virus was inoculated intravenously into rabbits. These results are shown in text figure 1. The guinea pigs not killed for passage were held for serologic studies.

There was no significant difference between the effects on guinea pigs of the bovine-passaged and lapinized forms of rinderpest virus. It can be seen in table 1 that

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The monthly reports occasionally referred to in this publication are official reports on the progress of the project, rendered monthly to the U. S.-Canadian Commission and filed in the Office of the Chief of Chemical Warfare. Interim Reports are special reports on important scientific accomplishments and developments in the project and rendered at such times as developments and circumstances warranted. Most of these Interim Reports constitute the basis of the present publication.

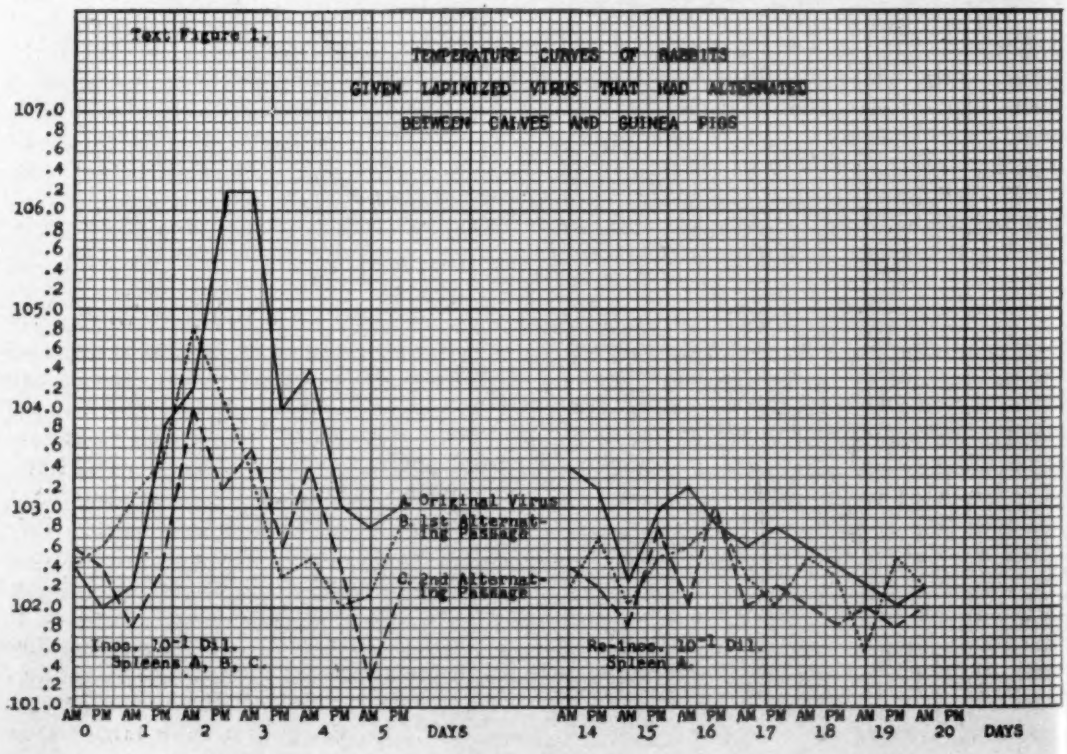
TABLE I—Study of Rinderpest Virus in Guinea Pigs by Serial Passage and by Alternating Passage with Calves

Calf series	Guinea pig passage	Signs in guinea pigs	Titration of guinea pig spleen in calves
Lapinized Virus			
A	1	None	10^{-3} showed rinderpest 10^{-4} no rinderpest
	2	None	10^{-1} showed rinderpest 10^{-2} not tested
	3	None	10^{-1} no rinderpest
B	1	None	10^{-1} showed rinderpest 10^{-2} not tested
	2	None	10^{-1} not tested
C	1	None	10^{-1} not tested
Bovine-Passaged Virus			
A	1	None	10^{-2} showed rinderpest 10^{-3} no rinderpest
	2	None	10^{-1} showed rinderpest 10^{-2} not tested
	3	None	10^{-1} no rinderpest
B	1	None	10^{-1} showed rinderpest 10^{-2} not tested
	2	None	10^{-1} not tested
C	1	None	10^{-1} not tested

virus was established in guinea pigs in the first passage and that it was transferred for a second, but not for a third, serial passage. No marked signs were produced in guinea pigs and since only slight and occasional temperature elevations occurred, the demonstration of virus depended upon inoculations into calves. The concentration of virus in spleens from guinea pigs of the first passage caused illness in calves at a 10^{-2} dilution for the bovine-passaged and a 10^{-3} dilution for the lapinized form, but not at higher dilutions.

Transference of the lapinized virus through one alternating passage in guinea pigs and calves caused a marked decrease in the pathogenicity for rabbits, and after a further alternating passage only a slight rise in temperature was noted in rabbits given the virus.

Neutralization Tests. — As mentioned above, guinea pigs given the original passage of bovine virus and lapinized virus as well as two further serial transfers of each were used for neutralization studies. In



addition, other guinea pigs were given intravenously 1 cc. of avianized egg vaccine[2] that represented virus carried serially in eggs for 72 and 80 passages. All animals were held twenty-one days and then bled for serum. Neutralization tests were done in rabbits by the method employed for the study of calf serums.[3] The results are presented in table 2.

TABLE 2—Neutralization Tests in Rabbits with Serums from Guinea Pigs Inoculated with Various Forms of Rinderpest Virus

Virus used as inoculums	No. of serial passages in guinea pigs	Neutralization tests in rabbits 10-100 m.i.d.'s virus
Bovine-passaged virus	A 1	Neutralization
	A 2	Neutralization
	A 3	No neutralization
Lapinized virus	A 1	Neutralization
	A 2	Neutralization
	A 3	No neutralization
Avianized virus	A 1	No neutralization
Uninoculated		No neutralization

Excellent correlation is shown between the establishment of virus in guinea pigs and the development of neutralizing antibodies; that is, animals in the first and second serial passages contained virus in their spleens, and those similarly inoculated had in their serums a neutralizing capacity for 10 to 100 m.i.d.'s of lapinized virus. Serums of uninoculated guinea pigs and those given the avianized vaccine did not neutralize.

DISCUSSION

It is interesting to note that passage of the lapinized virus through guinea pigs and calves in an alternating series caused a rapid deterioration of its pathogenicity for rabbits. Only one passage was necessary to bring about a marked decrease. Since it has been shown[1] that a calf passage has no effect, this alternation in the property of the lapinized form can be attributed only to the guinea pig passage. It might appear that the virus originally inoculated remained residual in the guinea pig. If that were so, however, it should have remained unaltered in its effect on rabbits.

Another observation that bears interest in a comparative way is the apparently greater susceptibility of guinea pigs than rabbits in the first and second serial passage from calves infected with bovine-passaged virus. Neither animal showed signs of illness but, as previously reported,[1] the virus concentration in spleens from rabbits of the first passage as measured by calf infectivity tests was 10^{-1} whereas, as shown above, the virus concentration in spleens from guinea pigs measured $10.^{-2}$ Furthermore, there was no virus shown to be present in spleens from rabbits of a further passage but, in guinea pigs, virus was present in their spleens. However, after one alternating passage of virus between rabbits and calves the picture changed, and it then produced a high thermal reaction in rabbits, whereas, guinea pigs showed only slight and occasional temperature elevations even after two alternating passages.

Even though no definite signs of illness were shown by guinea pigs, the data discussed above suggested that rinderpest virus was established in them and that it caused infection in the usual sense of that word. The conclusion that infection occurred in guinea pigs was permitted by the production of neutralizing antibodies. That this was not the result of giving a certain mass of virus, but occurred from infection and subsequent multiplication of that mass, is shown by the administration of a similar dosage of the avianized form of rinderpest virus. The avianized form produced no neutralizing antibodies.

Unfortunately, the original purpose of providing a useful experimental animal failed, since no definite signs of illness resulted from infection. Since the avianized form did not produce neutralizing antibodies in guinea pigs, neutralization tests in rabbits with such serums as a means of checking vaccines prepared from eggs is impossible. However, the biologic properties shown by the virus of rinderpest in this study are most interesting, in so far as they clearly demonstrate that it can be modified in pathogenicity unusually quickly, and that it reacts quite differently, depending on the host cell.

SUMMARY

Rinderpest virus in three forms—bovine-passaged, lapinized, and avianized—was given intravenously to guinea pigs. The avianized virus caused no signs of illness and neutralizing antibodies were not produced. The lapinized and bovine-passaged types of the virus also caused no signs of illness, but were established and passed serially once as could be demonstrated by inoculations into calves. It was found that the lapinized form was markedly altered in its pathogenic property by one guinea-pig passage. As discussed in the text, these results suggested that the virus caused infection in the usual sense of that word. Furthermore, neutralizing antibodies were demonstrated and it was concluded, therefore, that the guinea pig could be added to the list of laboratory animals susceptible to the virus of rinderpest.

References

[1]Eighth Interim Report.
[2]Twenty-fourth Monthly Report.
[3]Ninth Interim Report.

Rinderpest

XI. The Survival of Rinderpest Virus in Various Mediums

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IT IS WELL KNOWN that viruses in general have more or less exacting environmental requirements for reproduction and survival. For many viruses, survival is possible only within relatively narrow limits of hydrogen ion concentration. In much work with rinderpest virus, it has been customary to employ unbuffered physiologic saline as the suspending medium. Although quite satisfactory, there is no data available to indicate that such saline is the best medium. The more that is known of the requirements of rinderpest virus, the more effectively it can be handled and used in the production of vaccine.

An investigation to determine the optimum hydrogen ion concentration and the optimum buffer salt concentration for rinderpest virus has been completed within sufficiently narrow limits for our practical requirements.

The pH study was made by determination of the relative survival time of 10^{-2} dilutions of rabbit-adapted rinderpest virus in M/10 phosphate buffers at various hydrogen ion concentrations when held at 36 F. Virus suspensions in M/10 phosphate buffers at pH 6, pH 7, and pH 8 were tested and, in turn, compared with virus suspended in neutral distilled water, neutral physiologic saline, and normal eleven-day egg fluids at their unaltered pH of 8.1 to 8.2. The neutral distilled water and the physiologic saline used in preparing virus suspensions for this work were freshly boiled and cooled under a soda lime filter to prevent reabsorption of carbon dioxide.

Sorensen's phosphate buffers have been employed wherever buffers were used. Sterile stock solutions of anhydrous dibasic sodium phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were prepared separately in the desired molar concentrations. The two solutions were then combined in proportions to give the required pH. The pH of each buffer was confirmed on a Beckman potentiometer to within 0.05 pH.

M/10 phosphate buffer in a range of pH 6 to pH 8 was selected as a starting zone because physiologic saline, buffered to about pH 7 with phosphate buffer salts, has helped to prolong the antigenicity of the bovine tissue vaccine. Likewise, physiologic saline, which has a molarity near M/10, *i.e.*, at M/6.88, has been found a reasonably satisfactory, though not ideal, medium for the virus.

The virus employed had undergone four alternating calf-rabbit passages, with six rabbit passages (D-VI) prior to the last calf 676. The spleen of calf 676 frozen in Petri dishes at —5 F. was the source of all virus used. When freshly suspended in physiologic saline or M/10, pH 7 phosphate buffer, a 10^{-4} dilution of the spleen of calf 676, will produce temperatures above 104.5 F. in normal rabbits. All virus suspensions used for these tests were stored in 10^{-2} dilutions at 36 F. When 10^{-3} or 10^{-4} dilutions of the virus were used for rabbit inoculation, they were prepared from the 10^{-2} dilutions just prior to use. All rabbits were inoculated intravenously with 1.0 cc. of the dilution indicated.

The results of these tests for the survival of the virus are shown in table 1. The suspending solutions tested have been listed in the order of their ability to maintain the virus in a sufficiently active state to produce temperatures above 104.5 F. in normal rabbits.

It is evident from table 1 that pH 7, M/10 phosphate buffer is the least injurious to the virus of any of the solutions tried. The pH6 buffer was nearly as good for maintaining virus activity as was the

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pH 7. A comparison of the results of 10^{-3} and 10^{-4} dilutions at pH 6 and pH 7 strongly suggests that the exact optimum lies somewhere between pH 6.5 and pH 7. Physiologic saline and distilled water at pH 7 were less injurious to the virus than either phosphate buffer at pH 8 or normal egg fluids at pH 8.

TABLE 1—Survival of Virus at 10^{-3} Dilution in Various Solutions at 36 F.

Order of value	Solution	Dilution tested	Days survived	Days did not survive
I	Phosphate buffer pH 7-M/10	10^{-4}	18	21
		10^{-3}	30	36
		10^{-2}	44	48
II	Phosphate buffer pH 6-M/10	10^{-4}	14	18
		10^{-3}	22	26
		10^{-2}	44	50
III	Physiologic saline pH 7-M/6.88	10^{-4}	6	10
		10^{-3}	14	18
		10^{-2}	26	30
IV	Distilled water pH7	10^{-4}	..	2
		10^{-3}	10	14
		10^{-2}	18	22
V	Phosphate buffer pH 8-M/10	10^{-4}	1	4
		10^{-3}	..	14
		10^{-2}	10	14
VI	Normal 11-day egg fluids pH 8.1-8.2	10^{-4}	..	1
		10^{-3}	1	5
		10^{-2}	5	14

In routine passage of rinderpest virus in eggs, we have been passing mixed allantoic and amniotic fluids only for yolk sac-inoculated eggs; and chorioallantoic and amniotic membranes suspended in the homologous allantoic and amniotic fluids for membrane-passage eggs. Such egg fluid suspensions as were not used for immediate passage have been stored frozen. It is now apparent that these fluids at their natural pH 8 provide a relatively poor medium for the storage of the virus. It may well be that the addition of phosphate buffer to adjust the pH to 7 or the inclusion of yolk, which is normally about pH 7, will help maintain virus activity for longer periods than do egg fluids alone. Experiments to determine the relative effect of adding these buffer substances are under way.

With this knowledge of the pH requirements of rinderpest virus, the need for including a buffer in the avianized virus vaccine became more apparent. The available literature and our experience in the desiccation of biologic materials indicate that natural protein buffers are preferable to inorganic salts for this purpose. Experiments are being conducted using skimmilk and egg yolk as protective mediums for drying virus suspensions from the frozen state.

MOLARITY

For some viruses, notably influenza, it has been shown that the concentration of salts in the suspending medium is as important to the maintenance of virus activity as is the pH of the medium. Having determined the approximate optimum pH for this virus at an arbitrary one-tenth molarity, virus survival trials were then made to determine the most favorable molarity within a tenfold dilution range of from M/1 to M/1,000 phosphate buffer.

The method employed was the same as for the pH determinations except that, in this instance, all buffer solutions were at pH 7 with the buffer salt concentration being the variable. Fourth alternating passage rabbit-adapted virus in the form of frozen spleen of calf 676 was ground and diluted in pH 7 phosphate buffer solutions of M/1, M/10, M/100, and M/1,000. A 10^{-3} dilution of each suspension was then stored at 36 F. After various time intervals, the suspensions were tested in normal rabbits by the intravenous administration of 1.0 cc. In all instances, when further dilutions were made beyond 10^{-2}, they were made

TABLE 2—Survival of the Virus in pH 7 Phosphate Buffers of Different Molarities

Order of value	Solution molarity	Dilutions tested	Days survived	Days did not survive
I	M/10	10^{-3}	22+	..
II	M/100	10^{-3}	..	14
		10^{-2}	18	..
III	M/1	10^{-3}	10	14
IV	M/1,000	10^{-3}	..	14

with the homologous buffer solution just prior to injection.

Tests were made on only enough rabbits to determine the relative value of the four salt concentrations, without trying to determine the exact end point of virus survival for each solution. Results of these tests are shown in table 2, in which the solutions are listed in the order of their ability to maintain virus activity capable of inducing positive temperatures in normal rabbits.

It is apparent from the tabulated results that M/10 buffer salt concentration is the best of the four concentrations tested. The fact that the virus failed to survive fourteen days in a 10^{-2} dilution of M/1,000, pH 7 buffer is in agreement with the inability of neutral distilled water to support virus activity as well as does saline or M/10, pH 7 buffer solutions. Not only did the M/1 concentration of buffer salts have a relative inhibitory effect on virus survival, but it coagulated the spleen protein into a tenacious mass with which it was difficult to work.

SUMMARY

By determination of the ability of a 1.0 per cent suspension of rinderpest virus to survive in various suspending mediums at 36 F., the following facts have been revealed:

1) The stability of this virus can vary considerably, depending upon the pH and salt concentration of the suspending mediums.

2) In M/10 phosphate buffer, a suspension of rinderpest virus survived at pH 7 several days longer than at pH 6, and nearly five weeks longer than at pH 8.

3) Under comparable conditions, the virus survived much longer in an M/10 salt concentration than in M/1, M/100, or M/1,000 concentrations.

4) For maintaining the activity of rinderpest virus in a 10^{-2} suspension, an M/10 phosphate buffer at pH 7 was definitely superior to neutral physiologic saline, neutral distilled water, or normal eleven-day egg fluids at pH 8.

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XII. The Successful Use of Young Chicks to Measure the Concentration of Rinderpest Virus Propagated in Eggs

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CONTINUOUS serial passage of rinderpest virus in eggs by the yolk sac route causes an alteration in its pathogenicity for calves,[1] and many calves show no signs of illness when given the virus, while others show an elevated temperature only. Therefore, an immune response to pathogenic virus is necessary to determine infection. Supplementary and conclusive evidence is found when neutralizing antibodies are demonstrated.[2] This change in rinderpest virus by avianization has been fortunate because it permits the production of vaccine[3] containing active virus. Since avianized virus used in the production of vaccine produces no signs of infection when inoculated into embryonated eggs, the demonstration of activity depends on the effects produced in calves. However, the calf is a large animal and this procedure is not only costly but imposes limitations on the amount of work that can be done with this form of virus.

A study of rabbits,[4] guinea pigs,[5] and mice,[6] showed that they are infected and develop neutralizing antibodies when given natural and lapinized forms of rinderpest virus, but only when large amounts are given. Neutralizing antibodies are not formed when vaccine containing avianized virus is inoculated and, either the virus is altered, or concentrations are not sufficient to overcome the threshold of infection in these animals.

Previous studies[7] had revealed that chicks inoculated by various routes with virus propagated for several passages on the chorioallantoic membranes of embryonated eggs contained virus in suspensions of their pooled organs, as shown by inoculation in calves. Also, chicks hatched from infected eggs contained virus in their body organs for several days, and their serums, twenty-eight days after hatching, neutralized virus that was pathogenic for calves. It seemed worthwhile to extend the study of small animals to include chicks, and similar experiments were made with them. Fortunately, their susceptibility to avianized virus equalled that of calves, thereby making it possible to determine the presence and concentration of virus in vaccines. Furthermore, fundamental investigations need no longer depend on the use of calves.

EXPERIMENTAL STUDIES

In all the work with chicks reported herein, lyophilized avianized virus (vaccine) was used. The vaccine was reconstituted with sterile distilled water, centrifuged for five minutes at 1,000 r.p.m., and dilutions of the supernatant made in physiologic saline were used for inoculation. In early tests, dilutions of 1:40 were used, since greater concentrations frequently were toxic, causing death a few minutes after inoculation. Later, it was shown that dilutions of 1:100 were satisfactory and thereafter this dilution was used routinely.

Chicks not over 1 day of age were inoculated regularly and easily, but greater difficulty was encountered in older chicks. Groups of 3 chicks were inoculated intravenously in the medial metatarsal vein with 0.5 cc. of virus suspension as prepared

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The monthly reports occasionally referred to in this publication are official reports on the progress of the project, rendered monthly to the U. S.-Canadian Commission and filed in the Office of the Chief of Chemical Warfare. Interim Reports are special reports on important scientific accomplishments and developments in the project and rendered at such times as developments and circumstances warranted. Most of these Interim Reports constitute the basis of the present publication.

above. Compression of the vein at the hock was accomplished with the index and forefinger, while the toes were grasped with the thumb and little finger of the same hand; the other hand manipulated the syringe. Restraint of the chick was accomplished by rolling in a towel with the limb to be inoculated extended from the towel.

All of the chicks were held for an arbitrarily chosen period of twenty-one days after inoculation, and then bled for serum samples by heart puncture. However, in a study of serums obtained at 14-, 21-, and 28-day intervals after inoculation was made, it was found that no antibodies were present after fourteen days, while after twenty-one days about 85 per cent of the serums neutralized 10 to 100 m.i.d.'s of virus, and after twenty-eight days the serums neutralized at least tenfold more virus than at twenty-one days. Most of the tests of serums consisted of conducting neutralization experiments in rabbits by a modification of the method previously described. In the original tests, results were based on the reaction shown by rabbits to the test mixture of virus and serum. This is now considered presumptive and the conclusion is based on the response of rabbits over a five-day period to a challenge dose of virus given in similar concentration eight or nine days after the initial injection. Thus, four types of reaction are possible: (1) no elevation in temperature shown to initial inoculation but increases on challenge—neutralization; (2) increased temperature to initial inoculation and none after challenge—no neutralization; (3) no increase in temperature following either inoculation—doubtful, and can be interpreted as either partial neutralization or insusceptibility to the dosage of virus given; (4) increase of temperature after each injection—doubtful, and probably represents intercurrent infection. Obviously, the first two were usual, while the third occurred in approximately 5 per cent of inoculated animals, and the fourth was extremely rare, occurring only once in several hundred tests. Serums showing these doubtful reactions at first generally give a positive or negative neutralization when retested.

Other observations, largely empiric at present, indicate that all test serums should be held for two or three days before testing. Serums from some calves immunized by vaccination failed to neutralize when freshly drawn, but neutralized after a period of storage in the refrigerator. This has not been observed for prevaccination serums, or serums from animals that were not immune. Apparently, this effect is not confined to bovine serums alone, as this phenomenon has been observed in the serums from guinea pigs, mice, and young chickens. Pregnant rabbits were not used, as they tended to give a doubtful test; that is, no thermal response to either inoculation.

A total of 35 lyophilized vaccines were tested in chicks, 30 of which produced immunity and neutralizing antibodies in calves and five that elicited no such response. These results are shown in table 1. From

TABLE I—Comparison of Calves with Chicks as Test Animals for the Presence of Rinderpest Virus in Avianized Vaccines

	Results in calves			Results in chicks
Vac. No.	Response to test virus		Effect of post-vac. serum against 10-100 m.l.d.'s virus in rabbit test	Effect of post-vac. serum against 10-100 m.l.d.'s virus in rabbit test
30	30	Immune	30 Neutral.	25 Neutral. *5 No neutral.
5	5	Nonimmune	5 No neutral.	5 No neutral.

*On retest these 5 neutralized, therefore giving a 100 per cent correlation with calves.
Vac. = vaccine.
Neutral. = neutralization.

the vaccines that immunized calves, five were chosen for titration purposes. Tenfold dilutions were prepared and inoculated into both calves and chicks. Serum was collected from the chicks twenty-one days after inoculation and from the calves fourteen days afterwards. The calves then were given, subcutaneously, virus pathogenic for normal calves. These results are presented in table 2.

It can be seen in table 1 that the correlation between calves and chicks in demonstrating the presence or absence of virus in vaccines is 85 per cent. This degree of correlation was obtained also when the

amounts of virus were determined as shown in table 2; that is, for four vaccines there was perfect agreement between the results obtained from calves and chicks. The vaccines that failed to produce neutralizing antibodies in chicks but did in calves gave perfect agreement when retested. It should be noted that the five vaccines which failed to immunize calves produced no neutralizing antibodies when inoculated into chicks.

TABLE 2—Comparison of Calves with Chicks as a Test for Amount of Rinderpest Virus in Avianized Vaccines

		Results in calves		Results in chicks
Vaccine No.	Dilution tested	Response to test virus	Effects of post-vac. serum against 10-100 m.i.d.'s virus in rabbit test	Effects of post-vac. serum against 10-100 m.i.d.'s virus in rabbit test
66	10^{-2}	Immune	Neutralization	Neutralization
	10^{-3}	Nonimmune	No neutralization	No neutralization
67	10^{-2}	Immune	Neutralization	Neutralization
	10^{-3}	Nonimmune	No neutralization	No neutralization
78	10^{-2}	Immune	Neutralization	Neutralization (?)
	10^{-3}	Immune	Neutralization	No neutralization
78A	10^{-2}	Immune	Neutralization	Neutralization
	10^{-3}	Immune	Neutralization	Neutralization
82	10^{-2}	Immune	Neutralization	Neutralization
	10^{-3}	Nonimmune	No neutralization	No neutralization

Vac. = Vaccine.

DISCUSSION

The results show that chicks can be substituted for calves in tests for the presence and concentration of rinderpest virus propagated in eggs. This finding permits a means of checking vaccines prepared from eggs without the use of calves as test animals. Furthermore, many fundamental investigations of this form of virus can be conducted that otherwise would be limited, since the calf is a large animal and requires a relatively great amount of living room and care.

The discrepancy between the results reported for calves in comparison with chicks needs elucidation. Apparently, several explanations are possible which are concerned with the chick rather than the vaccines, since on retest they produced neutralizing antibodies. It may be that the technique was faulty, but this is unlikely for obvious reasons. Certain chicks may be insusceptible to the virus; that is, if the virus must multiply in the chicks in order to incite the formation of antibodies. However, the most likely explanation lies in the interval between inoculation and bleeding, since after a 14-day period no antibodies are present and there is an increase at twenty-eight days over the 21-day period. The arbitrarily chosen period of twenty-one days used for the tests reported herein probably represents that period when antibodies are forming, and in those serums that did not neutralize, a sufficient level had not been reached. It is suggested, therefore, that the method be changed from a 21-day period between inoculation and bleeding to twenty-eight days.

SUMMARY

Serums were obtained from chicks inoculated intravenously with avianized rinderpest virus that had been lyophilized (vaccine), and then tested for neutralizing capacity against lapinized virus by the rabbit method. It was found that neutralizing antibodies developed when vaccines containing active virus were given, and when various dilutions were tested, the results paralleled those obtained with calves. It was concluded, therefore, that the chick substituted for the calf satisfactorily, when it was desired to determine either the presence or concentration of the avianized form of rinderpest virus.

References

[1]Seventh Interim Report.
[2]Ninth Interim Report.
[3]Twenty-fourth Monthly Report.
[4]Eighth Interim Report.
[5]Tenth Interim Report.
[6]Unreported.
[7]Sixth Interim Report.

Rinderpest

XIII. The Production of Rinderpest Vaccine from an Attenuated Strain of Virus

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THE INVESTIGATIONS of Shope and his coworkers[1,2,3] demonstrated that rinderpest virus may be cultivated in embryonating eggs and that, under certain conditions, there is a marked attenuation of the virus for bovine animals. He has also demonstrated that the attenuated virus produces a solid immunity in bovine animals against fully virulent spleen virus, and that the mild reaction to the vaccine is not contagious.

Before the attenuated virus could be accepted as a "live" vaccine for use in the field, it was necessary to confirm the assumption that the mild symptoms produced by the vaccine could not be transmitted to other calves by contact. Shope[3] had already conducted experiments with 2 calves which indicated that the attenuated virus was not contagious. In order to confirm this finding, 10 calves were vaccinated with the attenuated virus and placed in a large open ward together with 4 normal, unvaccinated animals. A common drinking trough was provided which was not cleaned during the entire experiment, the consumed water being replaced as it was used. Individual feed boxes were provided but all animals had access to each box. Close and intimate contact was furthered by the fact that the calves were of both sexes and some of the females were in estrus. This resulted in considerable milling around and excitement among the animals during the entire period covered by the experiment. The normal, unvaccinated calves remained normal throughout the contact period. Five of the vaccinated calves demonstrated an elevation of temperature within five to ten days following vaccination, which persisted from two to six days. After twenty-three days of contact, a normal calf was added to the group as a virus control and all animals received an injection of fully virulent spleen virus subcutaneously. All of the vaccinated calves were shown to be immune while the contact animals and the control calf promptly developed the disease and either died or were destroyed at the point of death. It is believed that this experiment provides sufficient evidence that the mild symptoms produced by the attenuated vaccine are not transmitted to other calves by close contact.

The next consideration of the attenuated virus led to an experiment to determine whether the attenuation was fixed or would revert to its original bovine pathogenicity by repeated blood passages. Blood was drawn from a calf five days after injection of the attenuated virus and injected into a normal animal. When this second calf demonstrated an elevation of temperature, blood was transferred from this animal to a third calf and the same procedure followed with subsequent animals. As the blood passages progressed there was, in general, a shortening of the time interval between injection of the blood and demonstration of an elevation in temperature. None of the blood-passage calves were seriously ill until the sixth blood passage demonstrated a marked thermal reaction (107.0 F.) on the fourth day after injection and died of the disease on the tenth day. At the time this calf was inoculated, a normal calf was placed in the same pen in intimate contact. The contact calf developed the disease ten days after the first

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high temperature response in the inoculated animal and recovered, following a serious illness of seven days. All the blood-passage animals were found to be immune when challenged with virus. This experiment demonstrated that the blood of animals, which receive inoculations of the attenuated virus, contains rinderpest virus of the same mild, attenuated type as that in the egg cultures. It also shows that repeated blood passages of the attenuated virus may cause it to revert to its original bovine pathogenicity after six or more passages made in this manner. Thus, where there is a shortage of the attenuated virus vaccine, the blood of a vaccinated calf could be used to vaccinate additional animals but it would not be without danger if this process were repeated, using the blood of animals vaccinated with the blood of other animals. It is also demonstrated that the vaccine is perfectly safe to use in the field, since wholesale transmission of blood from animal to animal is not a situation which is liable to occur. It is suggested that all of the animals in one vicinity be vaccinated at approximately the same time, leaving no susceptible animals to act as blood-passage calves.

AVIANIZED SEED VIRUS FOR VACCINE PRODUCTION

Recent investigations of the adaptation of the virus to growth in eggs and attenuation of the virus for bovine animals indicate that little or no attenuation is obtained by repeated passages on the chorioallantoic membrane. Extended transfers by this route, as far as the ninetieth serial passage, maintain the virus in sufficient virulence to produce the disease in a fatal form when injected subcutaneously in a calf. On the other hand, transfer of the virus by way of the yolk sac results in a rather rapid attenuation of the virus for bovine animals.

It has been reported by Shope[2, 3] that a number of preliminary transfers of the virus on the chorioallantoic membrane are necessary before the virus is adapted to grow in eggs when transferred by way of the yolk sac. The manner in which the chorioallantoic transfers are made has been described,[1] and the only additional information on this subject indicates that the cultures should be incubated a minimum of seventy-two hours in 10-day embryonated eggs. A shorter incubation period results in a loss of the virus, and there is no advantage to be gained by prolonging the incubation period more than three days.

The number of virus passages on the membrane which are required before the virus is sufficiently adapted to grow in eggs, when transferred by way of the yolk sac, varies with each series of egg cultures. In some series, the eighth membrane passage is sufficiently adapted while, in others, 15 or more passages on the membrane are required. Other strains of rinderpest virus may require still more extended passages on the membrane before they become adapted. For example, the caprine strain of virus was not adapted after 19 membrane passages but was capable of growth after 37 such passages. The Indian strain was not sufficiently adapted to grow in the yolk sac after 30 membrane passages, at which point the series was discontinued. The North African strain, for some reason, could not be successfully transferred in eggs by way of the chorioallantoic membrane.

Since there is no macroscopic evidence of infection in eggs which have been inoculated with virus, transfer material must be tested in calves at regular intervals to prove its presence. It has been customary to test the membrane-passage material by calf inoculation after four transfers and again at the time an attempt is made to transfer the culture by way of the yolk sac. When it is necessary to hasten the production of attenuated virus, it is possible to start a side series of yolk sac cultures after eight or ten membrane passages, continuing the membrane passages as well to avoid loss of time. If the virus fails to grow in the yolk sac at this level, another yolk sac series may be started at the twelfth or fifteenth membrane passage level.

Shope[2] has shown that the virus is not present in the combined allantoic and amniotic fluids of eggs inoculated by way of

the yolk sac after forty-eight hours' incubation of early passages. He also demonstrated that the virus is present in the embryo and mixed fluids after seventy-two or ninety-six hours' incubation. After a variable number of yolk sac passages, as few as 15 in some series, virus can be demonstrated in the mixed allantoic and amniotic fluids after twenty-four to forty-eight hours' incubation. This finally leads to the conclusion that further adaptation of the virus to growth in eggs occurs during yolk sac passages.

The number of yolk sac passages required to bring about the desired degree of virus attenuation is not exact. In one series, 19 yolk sac passages provided sufficient attenuation while in another series, 24 passages by way of the yolk sac were required to attenuate the virus to the point where it was suitable for vaccine purposes. Since different series of cultures are not consistent in this respect, the only way the degree of attenuation can be determined is by calf inoculation.

Attenuation of the virus by means of repeated transfers in the yolk sac of embryonating eggs is a progressive process. It is reasonable to assume that after numerous such transfers, the virus will become so altered or attenuated that it will no longer produce an immune response in calves. This characteristic of the attenuated virus has not been definitely determined. On one occasion, virus which had been carried through 58 consecutive passages in the yolk sac failed to produce immunity in a calf. However, the 53rd yolk sac passage from this particular series did produce immunity in several animals. This yolk sac series was started from the 27th membrane passage of the virus. The first 15 yolk sac passages were incubated seventy-two hours, the following transfers being made at 48-hour intervals. The failure of the 58th yolk sac-passage virus to produce immunity is probably due to excessive attenuation. The only alternative is that the virus was lost during transfer between the 53rd and 58th passage level. In any event, since the possibility does exist that the virus may become attenuated to the point where it will no longer produce immunity in cattle, a supply of low level passage virus should be maintained in a frozen state from which seed virus may be obtained for vaccine production.

An experiment was carried out to determine whether the virulence of attenuated virus from a high level yolk sac passage (49th) could be increased by one or two calf passages. It was hoped that the potency of the virus could be increased in this manner without losing the avianized characteristic or the ability to grow in eggs. A calf was inoculated subcutaneously with the attenuated virus and the spleen was removed when the first thermal response was observed. At the same time, blood was transferred from this calf to another animal. The spleen from the second calf was harvested when a thermal response was observed, which was also on the fifth day following inoculation. Suspensions of these two spleens were injected directly into the yolk sacs of 7-day embryonated eggs, but after four passages by this route, no virus could be demonstrated in the egg fluids. This failure led to an attempt to cultivate the virus from these spleens on the chorioallantoic membrane with the anticipation that virus from such a source could be adapted to culture in eggs more readily than bovine-passage virus. After several attempts, no virus could be demonstrated in the sixth membrane passage of these series.

The reason for the failure of virus from this source to grow in eggs is not known but it is believed that the amount of free virus present in the spleens of animals vaccinated with avianized virus is at such a low level that it is less than the amount required to establish the virus in eggs. It is possible that the immune response of the calf is responsible for reducing the virus level in the spleen.

All of the available evidence concerning the requirements for establishing the virus in egg culture indicates that a highly pathogenic strain of virus must be used and that the spleen used for this purpose must be harvested at the time when there is a maximum concentration of virus in that

organ. The concentration of virus in the spleens of calves inoculated with bovine-passage virus is greatest between thirty-six and forty-eight hours following the first rise in body temperature. At this time, the titer of virus in the spleen is at least 10^{-4} and occasionally as high as 10^{-5}.

PRODUCTION OF SEED VIRUS

Cultivation of rinderpest virus in embryonating eggs is started from 20 per cent suspensions of infected spleen in broth or physiologic saline solution. Bacteriologically sterile suspensions are placed on the chorioallantoic membrane of 10-day embryonated eggs in 0.2-cc. amounts. After seventy-two hours' incubation at 38 C., the infected portions of the membranes are harvested aseptically and ground in a glass grinder with 3 cc. of mixed allantoic and amniotic fluids. It has been shown that when this method of transfer is used, no virus is present in the mixed fluids. The fluids are utilized only as a vehicle for the infected membranes. The pooled membranes from several eggs are used to transfer the virus to the chorioallantoic membrane of the eggs of the following passage. A portion of membrane suspension from the fourth membrane passage is injected subcutaneously in a calf to confirm the presence of virus. Infected membranes from the tenth to the fifteenth passage level are ground in the usual manner and injected directly into the yolk sac of 7-day embryonated eggs in 0.5-cc. amounts. This transfer material from the membrane to the yolk sac should also be checked for the presence of virus by calf inoculation. As mentioned previously, the yolk sac-inoculated eggs are incubated ninety-six hours at 38 C. At the end of this period, the eggs are chilled forty minutes at —20 C. and the combined allantoic and amniotic fluids are collected for inoculation into the eggs of the next serial passage. After four passages by way of the yolk sac, the transfer material is again tested for virus content by calf inoculation. At the fifteenth yolk sac-passage level, an additional side series of yolk sac cultures is started, using an incubation period of forty-eight hours instead of ninety-six in order to hasten attenuation of the virus. When it has been demonstrated by calf inoculation that the series having the short incubation period contains virus, the series in which the longer incubation period has been practiced may be discontinued.

When a total of 20 or more transfers have been made by way of the yolk sac, the egg fluids may be tested to determine the degree of attenuation of the virus. A sufficient degree of attenuation is indicated when calves which have received 1 cc. of the mixed egg fluids subcutaneously do not become clinically ill within fourteen days and are subsequently immune to challenge with 100 minimal infective doses of bovine-passage virus. A temporary elevation of body temperature for two or three days may be expected and occasionally there may be a loss of appetite for a day. If the symptoms invoked by the avianized virus are more serious than those listed, and a definite illness is observed in the inoculated animal, more yolk sac passages are required to bring about still further attenuation.

STABILITY OF THE SEED VIRUS

The mixed allantoic and amniotic egg fluids which are used as seed for vaccine production rapidly lose their virus content when permitted to remain at room temperature (22 C.). The exact length of time the attenuated virus may be maintained at this temperature has not been determined, but there is evidence that exposure to room temperature for a period of twenty-four hours will result in complete loss of virus. Exposure of the infected egg fluids to a temperature of 60 C. for thirty minutes results in complete inactivation of the virus. The virus may be maintained in the fluids at a temperature of 2 C. for a period of from ten to fourteen days. If the fluids are promptly frozen immediately after harvesting, the virus remains viable for at least one year at a temperature of —20 C.

Attempts to dry the attenuated virus from the frozen state to preserve it for the subsequent egg passages have not been suc-

cessful. The dried egg fluids will invariably immunize a calf when reconstituted and injected subcutaneously, but will regularly fail to infect eggs when inoculated into the yolk sac. The failure of dried egg fluids to infect eggs is probably due to the loss of virus during the drying process.

VACCINE PRODUCTION

Frozen egg fluids from the 30th to the 50th yolk sac passage, containing virus which has been found to be sufficiently attenuated for calves, are used to prepare a supply of fresh seed virus by injecting 60, 7-day embryonated eggs by way of the yolk sac with 0.5 cc. of the stored fluids. The eggs are candled and placed on end, in a rack designed to hold 60 eggs, with the air cell end uppermost. The ends of the eggs are painted with tincture of iodine and a hole is drilled in the shell over the air cell. The frozen stock fluids are thawed and 0.5 cc. is injected into the yolk sac by means of an automatic syringe, to which is attached a 22-gauge needle, 1 1/4 in. long. The hole in the shell is closed by a mixture of warm beeswax and petrolatum, and the eggs are incubated in an upright position at 38 C. for seventy-two hours. At the end of this time, the eggs are candled again and those having dead or abnormal embryos are discarded. The eggs are placed in a freezer at a temperature of —20 C. for forty minutes; they are then removed to the refrigerator at a temperature of 2 C. until harvested. This chilling process appears to increase the yield of egg fluids and also permits harvesting the fluids with a minimum of blood. The tops of the eggs are painted with tincture of iodine and lightly flamed. The shell over the air cell is cut away with curved scissors. The inner shell membrane, the chorioallantoic membrane, and the amniotic membranes are opened with sterile, curved iris forceps to expose the fluids. The fluids are harvested by suction with a 30-cc. Luer syringe and a 19-gauge needle. A fine, wire gauze guard is provided for the tip of the needle to prevent plugging by the membranes. From 5 to 7 cc. of clear mixed fluids may be obtained from each egg in this manner. Allowing for the dead embryos, a conservative 250 cc. of fluids may be obtained from a tray of 60 eggs. The fluids are cultured on blood agar slants to ensure sterility and are then ready for use as seed. Since 0.5 cc. of seed is required for the injection of each egg to be used for vaccine, the seed fluids are diluted 1:10 with physiologic saline solution and thoroughly mixed prior to injection. As many eggs may be inoculated at one time as is convenient with the facilities at hand. However, since each lot of vaccine must be tested for potency, it is not practicable to inoculate less than 300 eggs for a lot. Vaccine production eggs are prepared for injection in exactly the same manner as for seed production. Injection of the diluted seed fluids is made in the same manner as described for seed production, but the eggs for vaccine production are incubated ninety-six hours instead of seventy-two. The production eggs are chilled by a forty-minute exposure to a temperature of —20 C. or by three or more hours at 2 C., after candling and discarding those containing dead or abnormal embryos. The eggs are transferred to individual blocks and the shell and membranes are opened in the manner described in the foregoing. The fluids are harvested by suction through a Pasteur pipette with a metal wire gauze guard over the end to prevent plugging. Suction is obtained by means of a Venturi water pump or an electrically driven vacuum pump. The fluids are collected directly into a sterile, 9-liter, pyrex bottle. After the fluids have been suctioned off, the embryos are removed with sterile forceps and placed in a sterile jar. When harvesting is complete, the fluids are measured, the embryos weighed, and the two components are milled together in an Eppenbach colloid mill. Milling is accomplished quickly, starting with a cold mill so that the mixture does not become warm during the process. A total of ten minutes is usually sufficient to bring the material to the desired degree of fineness. After milling, the mixture is drawn off in a 9-liter bottle and placed in the refrigerator at a temperature of 2 C. for a period of one or

two hours to permit the foam to break. By means of a sterile dispensing burette assembly, the vaccine is distributed in 20-cc. amounts in 60-cc. serum bottles and stoppered with sterile rubber stoppers. Immediately after filling, the contents are shell frozen by rotating horizontally in a bath of dry ice and alcohol. After freezing, the bottles are placed in the freezer at a temperature of —20 C. until the drying process is carried out.

If larger amounts of vaccine are desired in each container, the vaccine may be distributed in 500-cc. pyrex centrifuge bottles in 225-cc. amounts. These are stoppered and shell frozen in the same manner as the small bottles.

DRYING AVIANIZED VIRUS VACCINE

It has been shown that the undried vaccine retains its potency when stored at —20 C. but rapidly loses the virus content when exposed to room temperature. The potency of the fluid vaccine is also lost after a variable time up to fourteen days, at a temperature of 2 C. It is therefore necessary to remove the water from the vaccine to improve the keeping qualities so that it may be transported from the place of production to the point where it is to be used, without material loss of potency.

The process of drying from the frozen state has been known for many years. Among the first to describe the process were d'Arsonval and Bordes[4] in 1906, and Shackell[5] in 1909. However, it was only in the recent past, when human plasma was dried in large quantities for use in battle casualties, that the process has been extensively used in a commercial way.

Drying from the frozen state is accomplished by sublimation under high vacuum. The principal requirement in the drying process is that the water must be removed without producing any chemical changes of the constituents so that the biologic properties remain unchanged.

The broad requirements for drying by ice sublimation are apparent when the course of a particle of ice in the frozen material is traced through the course of drying. In the first place, the material must be exposed at all times to a pressure sufficiently low that water cannot exist in the liquid state. Second, heat must be applied to convert the particle of ice to water vapor. Third, the liberated water vapor must be permitted to escape from the frozen material. Finally, the water vapor must be removed from the system.

The degree of vacuum maintained in the system determines the temperature at which sublimation will occur. The water-vapor pressure is controlled by the efficiency of the condenser, the distance between the frozen material and the condenser, and the speed of the diffusion permitted by the tubes and connections between the frozen material and the condenser. The speed of diffusion is also influenced by the vacuum maintained in the system. The total pressure in the drying chamber during desiccation should be so low that sublimation of ice will take place below the melting point of the material being dried, approximately —8 C. in the case of this vaccine. The pressure in the drying chamber must be less than 500 μ of mercury, since the restriction of the bottle neck causes the pressure to be always higher in the bottle than in the drying chamber.

The drying system must be constructed vacuum tight and provided with a means of exhausting the air rapidly and effectively to 500 μ or less, in order that the frozen material does not melt. This can be accomplished by mechanical pumps of adequate size and capacity. For our work, we have found that the "Hypervac 20," or a pump having a larger capacity, may be used.

The function of the condenser is to remove the water vapor. The water vapor must be rapidly removed from the system, otherwise the vacuum will be lost and the material will melt. The condenser must be so efficient that the total pressure in the system will at no time during the process rise to a level which will permit melting.

In order to dry frozen material, heat energy must be applied to replace the specific heat of ice sublimation which is 675.4 calories per gram, at a temperature of —10 C.

to —30 C. If no external heat can reach the frozen material in a vacuum system with a cold condenser, an adiabatic state is produced, the temperature of the frozen material being reduced by evaporation to that of the cold condenser when an equilibrium is reached.

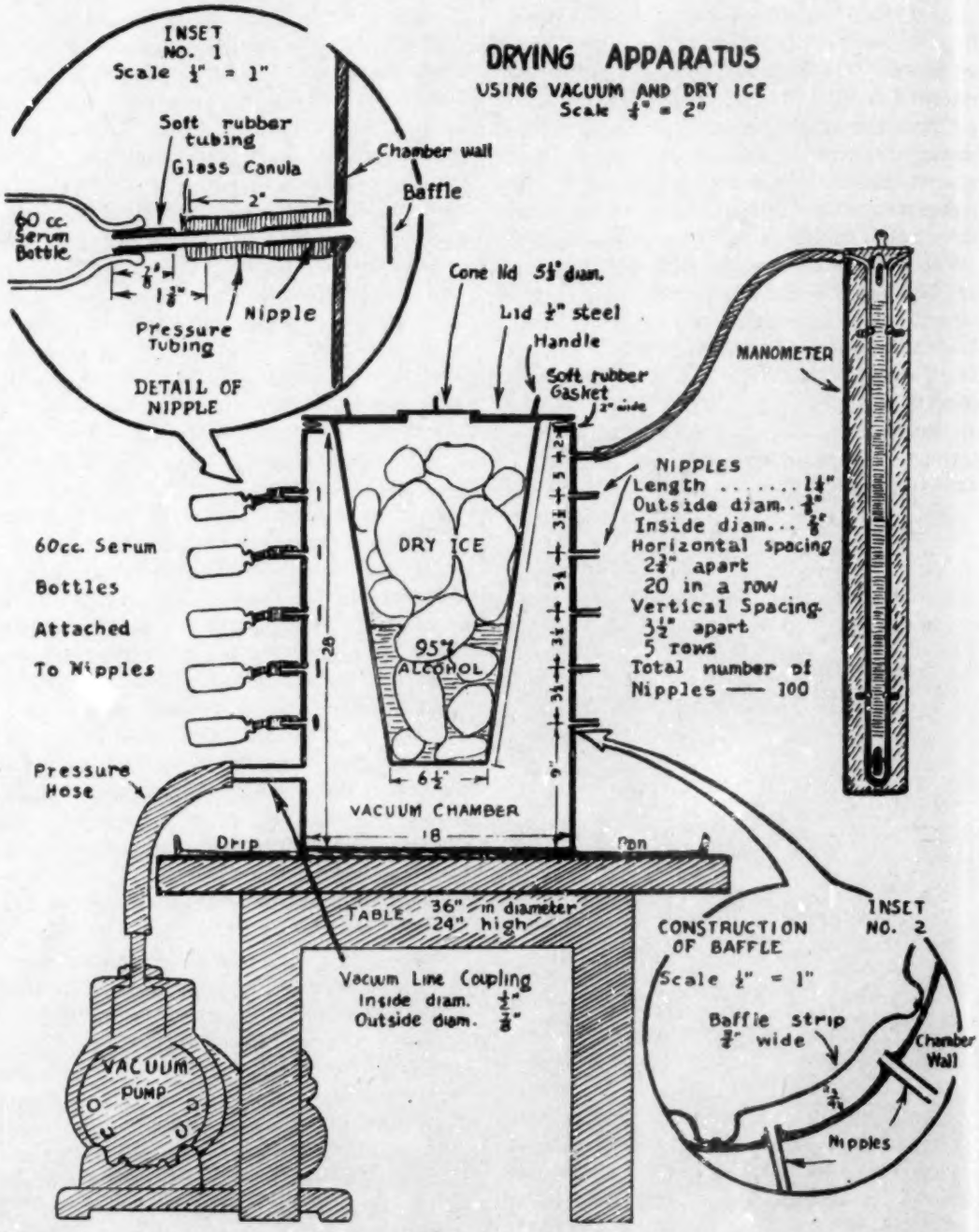
The diameter and, to a less degree the length, of the bottle neck and the connecting tubes, as much as any other factor, controls the rate at which a bottle of frozen material can be dried. The importance of this factor is clearly illustrated by applying the Knudsen equation, by which calculation of the flow of rarified gasses and vapors through tubes may be made.

$$G = 8/3 \cdot \pi \cdot R^3/L \cdot (P_1 - P_2 3 \cdot \sqrt{P½ \pi}$$

where G = flow of gas in cc. per second
R = radius of tube
L = length of tube
$P_1 - P_2$ = pressure difference
P = ratio of the density and pressure when the pressure is in dynes per square meter.

At low pressures, the flow of gas or vapor is proportional to the cube of the diameter of the tube. Thus, the volume of water vapor escaping from a bottle neck 20 mm. in diameter is eight times greater than that flowing from a bottle neck of 10-mm. diameter, under the same conditions. For this reason, containers having the largest neck openings should be used whenever possible. Otherwise, there is too much restriction to the free flow of water vapor and the pressure in the bottle may be raised to the point where melting will occur.

The first practical drying apparatus used by us was one constructed from a defective depth bomb casing obtained locally. This is shown in the illustration (text figure 1). One end was removed and a milled steel flange was welded in place. A truncated conical condenser was constructed and welded to a steel plate to fit the flange. A wide rubber gasket was placed between the flange and the condenser plate. Holes were drilled in the casing and threaded for nipples to which the bottles are attached by means of a special cannula. Baffles were installed inside of the casing opposite the nipples to prevent piling up of ice on the condenser. A mercury manometer, a McLeod vacuum gauge, and an efficient vacuum pump of large capacity complete the equipment necessary for drying. The glass cannulas with rubber gaskets in place are sterilized in boiling water in a tray constructed for the purpose. Alcohol is placed in the condenser to a depth of 8 in., and the condenser filled with CO_2 ice. The rubber tubes on the nipples are plugged and the vacuum pump started. When the pressure inside the drum has been reduced to 500 μ or less, the bottles may be attached. The tube on the nipple is clamped off, the plug removed, the stopper removed from the bottle, and a cannula inserted. The cannula is inserted into the tube on the nipple and the clamp is removed. There is considerable air in the bottle and the cannula, and when the clamp is removed, the pressure in the chamber will be raised. For this reason, the bottles must be placed in position for drying at regular intervals, permitting time for the pressure to be reduced to 500 μ or less as required. Otherwise, the pressure inside the bottles will be reduced to the extent that some slight, inapparent melting takes place and the result will be a hard, dense, flaky vaccine when dried. A relatively high humidity in the drying room has been found to be advantageous. Heat of crystallization, caused by condensation of water vapor upon the outside of the vials, increases the temperature inside the bottles and hastens sublimation. After approximately sixteen hours, heat is turned on in the drying room and the temperature gradually raised to 37 C. This temperature is maintained until just prior to removing the vials. The entire drying process requires approximately twenty-four hours. It is necessary that the condenser be kept filled with CO_2 ice during the entire time, and replacement is necessary at frequent intervals during the first six or eight hours of drying to compensate for the heat of crystallization of water vapor on the condenser. Approximately 200 lb. of CO_2 ice are required for shell freezing 1



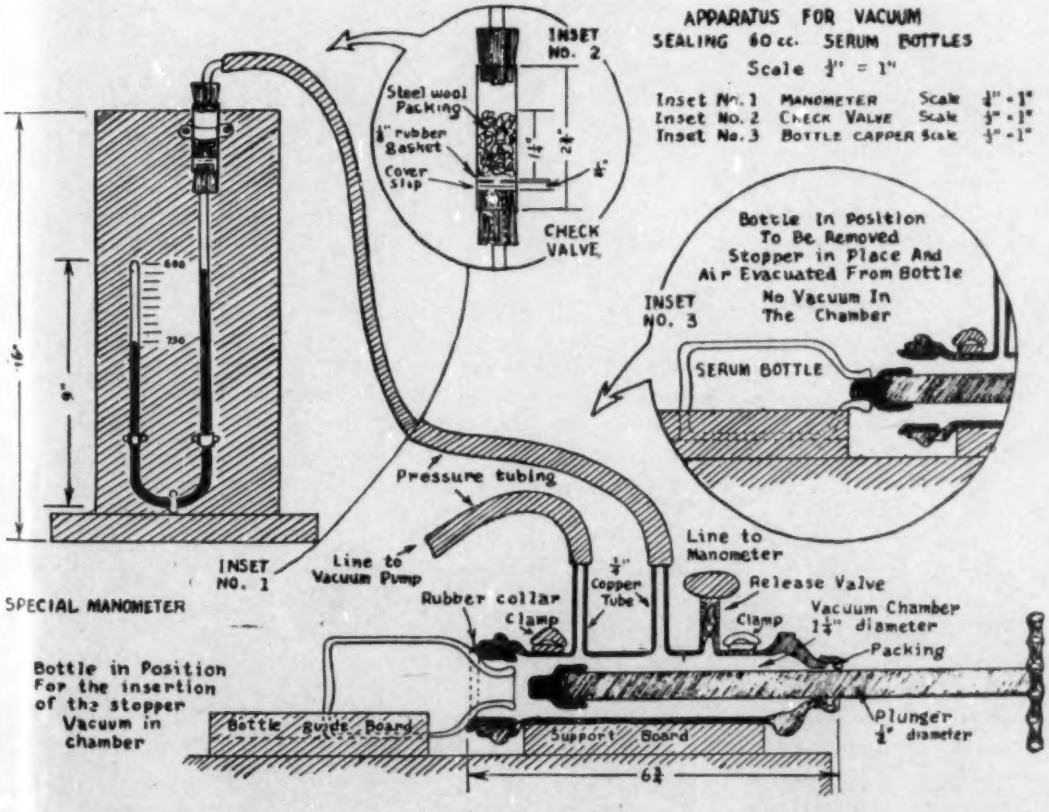
Text Figure 1

lot of vaccine and cooling the condenser for one 24-hour period.

At the end of the drying period, the vacuum pump is shut off and dry nitrogen is permitted to flow slowly into the drum by means of a tube connected to a nipple reserved for the McLeod gauge. The nitrogen flow should be regulated so that atmospheric pressure is not reached before a ten-minute interval. In this way, excessive turbulence is avoided and contamination of the vaccine held to a minimum.

The bottles are removed from the drum by clamping the rubber tube, removing the cannula and vial, and plugging the tube. The cannulas are placed in a tray for sterilization and the vials are closed with a diaphragm rubber stopped under vacuum by means of a device constructed for the purpose. The vacuum stoppering apparatus was constructed from plumber supplies obtained locally and is shown in the illustration (text figure 2).

A diaphragm type, skirted rubber stopper is placed on the end of the plunger, being held in place by the skirt. The end of the stopper is moistened with a small amount of sterile glycerin to facilitate insertion. The plunger is drawn back and the bottle is placed in position. When a suitable vacuum has been obtained, as indicated by the mercury manometer, the plunger is pushed in with a rotary motion to seat the stopper. Vacuum is relieved by means of the small relief valve, the plunger is pushed in all the way, and the stoppered bottle removed. The sudden release of vacuum would cause the mercury to rise rapidly in the manometer and break the end out of the glass tube except for the presence of the check valve. The check valve is constructed from glass tubing, rubber stoppers, and a



Text Figure 2

circular glass cover-slip. It is so arranged that air is permitted to enter the manometer at a reduced rate but with sufficient rapidity that accurate readings can be obtained on each bottle. This is accomplished by roughening, slightly, the surface of the rubber stopper on which the cover-glass rests.

The skirt of the rubber stopper is turned down as soon as the bottles are closed, and the whole stopper and bottle neck is immersed in a 10 per cent solution of isobutyl methacrylate (Dupont) in acetone. This solution provides a thin film, sealing the glass-rubber junction and preserving the rubber from oxidation. The vials are labeled with the lot number and date, and are packed in corrugated paper boxes containing 50 vials each. The vaccine is stored in the refrigerator at a temperature of 2 C.

The 500-cc. centrifuge bottles containing 225 cc. of vaccine may also be dried on the drum-type drier by using a 5/8 in. nipple on the drum and a special brass cannula to fit the tubing and a No. 6 rubber stopper in the neck of the bottle. A larger stoppering device, constructed in exactly the same manner as the small one, is used to stopper the large bottles. There is a special solid stopper for this type of container which has a heavy skirt and three holes containing small diaphragms.

Using a condenser as large as the one in the apparatus illustrated, it is possible to dry 300 small vials containing 20 cc. each of frozen vaccine. The drum provides for drying 100 vials at one run, so at least two drying runs may be performed before defrosting is necessary. The vaccine prepared from milled embryos and fluids contains about 3.5 per cent total solids. Thus, each 100 bottles will yield approximately 1,930 cc. of water which will be crystallized on the condenser. The surface of the condenser is approximately 2,700 sq. cm. The ice formed on the condenser is a good heat conductor, such completely de-aërated ice having heat conductivity of the same order as metals. There is only about 1 degree Centigrade difference in the ice temperature for each centimeter distant from the condenser surface up to a thickness of 5 cm. Application of these figures will reveal that a large number of vials may be dried before defrosting is necessary. We have frequently operated the apparatus successfully with 2 1/2 to 3 cm. thickness of ice on the condenser. When it is necessary to defrost the condenser, it is lifted out of the drum and the ice removed by sharp blows with a wooden mallet.

Early in 1945, a commercial experimental desiccator was obtained for drying the vaccine. The apparatus has a condenser which is mechanically refrigerated by a cascade system using propane and ethane as refrigerants. The bottles being dried are contained in a vacuum chamber and heat is applied by infrared heating elements. Space is provided for six 500-cc. centrifuge bottles which are placed in canisters containing the heating elements. The small vials are dried by placing them in a rack above a parabolic reflector with a single source of infrared heat at the focal point. A constantan copper thermocouple is inserted into one of the containers containing the frozen material and is connected to a recording potentiometer pyrometer through contacts installed in the glass door of the vacuum chamber. The pyrometer is mounted over a voltage regulator so as to permit connection between these two pieces of apparatus. The voltage regulator is motor driven and consists of a transformer having a stationary secondary and a rotatable primary coil. The pyrometer and voltage regulator are connected through a rocker arm, containing two microswitches, which follows a cam, fastened to the axle of the drive gear of the pyrometer. The instrument is constructed in such a way that the angular position of the axle of the pyrometer is representative of the temperature of the thermocouple. The two microswitches operate the motor of the voltage regulator so there is an increased current flow when the temperature of the thermocouple decreases and a reduced current flow when the temperature rises. By constructing a cam of proper dimensions, it is possible to apply any amount of infrared heat desired at any selected temperature, automatically. It is only necessary to determine (1) the num-

ber of watts required to dry the amount of material present, (2) the number of watts which may be applied at the various temperatures to insure a smooth, regular drying curve so that the material is not subjected to sudden changes of temperature during the drying cycle, and (3) the degree of final heat permitted by the material being dried without altering the biologic characteristics. The first two determinations may easily be made by operating the voltage regulator manually, keeping an accurate record of the wattage, time, and temperature of the thermocouple. This record is transferred to a graph and a cam constructed so that the proper number of watts will be applied at the suitable thermocouple temperatures. The third determination, the degree of heat which may be safely applied, can only be determined by experimental inoculation of animals.

It can be seen from the foregoing description that this apparatus is extremely versatile and may be utilized for the drying of almost any biologic material. It is possible to obtain residual moisture as low as

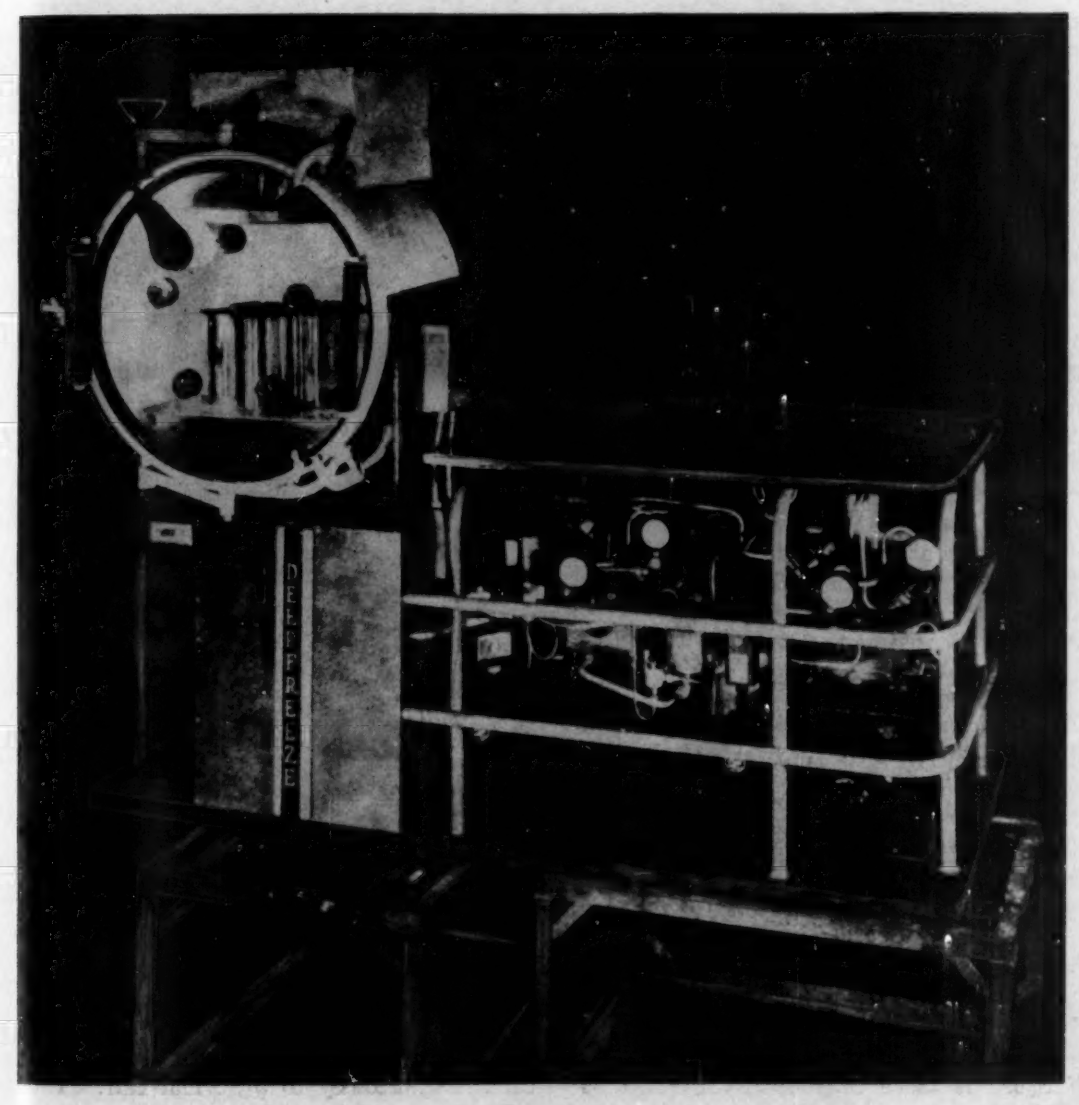


Figure 3

0.1 per cent, depending upon the material being dried. It is difficult to obtain moisture residuals of less than 0.5 per cent on the drum type drier with the same material in the same length of time.

It has been found that the attenuated rinderpest vaccine will maintain its potency equally well whether the residual moisture content is 0.7 per cent or 0.1 per cent.

Experiments to determine the maximum terminal heat permitted by the vaccine during drying have not established the exact point where total inactivation of the vaccine takes place. It is well known that biologic materials in general will withstand considerably more heat in a dry state under vacuum than when wet. The attenuated rinderpest virus, for example, is completely inactivated by exposure to a temperature of 60 C. for thirty minutes in a liquid state. Most of the vaccine lots dried by us have been dried with a terminal heat of 30 C. to 40 C. and having a terminal drying period at these temperatures of from four to six hours. We have found that these times and temperatures result in a product having a sufficiently low residual moisture content and which is still capable of producing a solid immunity in calves. The temperature of the vaccine in one run in the commercial desiccator accidentally went as high as 65 C., and was above 50 C. for more than one hour. When tested in calves, this lot produced a satisfactory immunity in a dilution of 10^{-2}. The efficacy of the vaccine in such a dilution indicates that very little of the vaccine potency had been lost by exposure to these high temperatures. It is shown that the vaccine is capable of withstanding much higher temperatures when the moisture is practically all removed than when in a wet state.

A few experiments have shown that the dried vaccine will maintain its potency in the presence of nitrogen equally well as when stoppered under vacuum. However, most of the vaccines produced here have been stoppered under vacuum since the process of reconstituting the dried vaccine for use is more conveniently carried out when there is a vacuum in the vial.

BACTERIOLOGIC STANDARDS FOR THE VACCINE

Bacteriologic control of the vaccine is maintained by using aseptic technique in all operations. Cultures of the egg fluids are made immediately after harvesting. The finished vaccine is cultured at the end of the bottling operation, and the dried product is reconstituted and cultures are made at this stage.

In spite of all precautions, it is almost impossible to obtain a completely sterile product from each lot of eggs. It is believed that some eggs contain bacteria when they are brought from the incubator for inoculation. It is well known that the egg shells carry large numbers of microörganisms. The most frequent organisms encountered as contaminants are members of the Escherichia group and staphylococci. Even in the case of vaccine lots showing relatively heavy contamination, no unfavorable results are observed when the material is injected subcutaneously in white mice or intraperitoneally in guinea pigs. The drying process operates to destroy a large percentage of the contaminants. In one lot of vaccine which contained contaminating organisms in a concentration of 900,000 per cubic centimeter, there was no growth on agar plates after drying and storage for fifty-two days at 2 C. Other lots containing 1,000 to 200,000 organisms were found to be sterile after drying and reconstituting. Thus, in an emergency, where vaccine is urgently required, in large quantities, it is believed that a slight amount of contamination with nonpathogenic organisms is not detrimental and that such vaccines may be safely used.

POTENCY TEST FOR VACCINE

Tests of the vaccine for potency are made routinely of each lot of vaccine. The most positive method of testing is by reconstituting one vial of the dried vaccine from each lot to a volume of 60 cc. with distilled water, physiologic saline solution, or phosphate buffer solution, and administering 0.5 cc. subcutaneously to a normal calf. The animal must fail to demonstrate any signs

of serious illness or reaction to the vaccine for a period of fourteen days. At the end of this time, the calf is given a challenge dose of bovine-passage virus (100 m. i. d.'s) subcutaneously, and if the animal remains normal for a period of eight days, it is considered to be immune and the lot is certified as satisfactory for use.

Details of other potency tests, immunologic reactions, and keeping qualities of the avianized rinderpest virus will be discussed in another article.

SUMMARY

1) Rinderpest virus, attenuated by culture in embryonated eggs, may be used as a vaccine to immunize cattle against the disease.

2) The mild symptoms which occasionally result from administration of the vaccine are not transmitted by close and intimate contact.

3) The attenuated virus does not regain its original bovine pathogenicity under normal conditions, and only experimentally, after a series of six direct blood passages in calves.

4) A method of producing a stock of attenuated seed virus is described.

5) Production methods for large quantities of the avianized vaccine are described in detail.

6) Methods of drying the vaccine to improve the keeping qualities of the product are described.

7) Bacteriologic and potency standards for the vaccine are briefly mentioned.

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* Filed in the Office of The Surgeon General, Army Service Forces, for security reasons, on the dates indicated, pending publication.

Rinderpest

XIV. Immunization Experiments with Attenuated Rinderpest Vaccine Including Some Observations on the Keeping Qualities and Potency Tests

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THE avianized rinderpest vaccine which we have prepared has been described in a previous paper.[1] This vaccine is based on the investigations of Shope and his coworkers,[2] which proved that the virus of rinderpest could be attenuated for bovine animals by repeated culture in eggs to the extent that it could be used safely as a vaccine.

Calves purchased locally or at the Montreal stockyards were used as experimental animals in nearly all the experiments conducted with the avianized vaccine. They were of various ages, from 3 months to 1 year, and consisted of both purebred and grade cattle. Many of them were from dairy breeds but a few beef breeds were represented. So far as we have been able to determine, all breeds are equally susceptible to the strains of rinderpest virus used for research studies.

During the immunization experiments, the calves were maintained in isolated units or stalls to prevent escape of the virus and to insure accuracy of results. These isolation units have been described in a previous paper.[3] Occasionally, intercurrent infections with pneumonia or parasitic infestations have obscured the results, but these animals have been omitted from the averages of compiled data.

One of the first considerations concerning the dried vaccine was the amount required to produce immunity. In the first experiment attempted to determine this factor, 10 calves were used. The vaccine used in the experiment had the following history: The seed virus used for production of the vaccine was the mixed allantoic and amniotic fluids from the 52nd yolk sac passage. The virus had originally been transferred on the chorioallantoic membrane for 27 passages. The first 15 yolk sac passages were incubated from seventy-two to ninety-six hours between transfers, and the remaining 37 yolk sac passages had been made at 48-hour intervals. The mixed fluids and embryos were harvested, milled, and shell frozen in 20-cc. amounts in 60-cc. serum vials in the usual manner. The material was dried under vacuum in the original containers from the frozen state. The dried material was reconstituted with distilled water to the original volume of 20 cc. just prior to use, and the 10 calves were vaccinated with various amounts of the vaccine as shown in table 1. The injections were made subcutaneously and 2 animals used for each dose. Temperatures were taken of all vaccinated animals twice daily following the vaccination.

TABLE 1—Effect of Various Amounts of Vaccine on Calves

Calf No.	Dose and dilution of vaccine	Highest post-vaccination temp.	No. of temp. readings over 104 F.	Result of challenge
435	1 cc. undiluted	106.0 F.	3	Immune
434	1 cc. undiluted	104.0 F.	0	Immune
432	1 cc. 1:2	106.0 F.	5	Immune
433	1 cc. 1:2	103.4 F.	0	Immune
430	1 cc. 1:10	103.8 F.	0	Immune
431	1 cc. 1:10	104.6 F.	2	Immune
428	5 cc. 1:100	105.0 F.	4	Immune
429	5 cc. 1:100	103.6 F.	0	Immune
426	1 cc. 1:100	103.6 F.	0	Immune
427	1 cc. 1:100	105.6 F.	6	Immune
453	Control	(Incubation period 3 days)		Died on 11th day

The vaccinated animals and an unvacci-

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nated virus control calf were given a challenge dose of virus (100 minimal infective doses) subcutaneously, twenty-three days after vaccination. All of the vaccinated animals were immune and the control calf died eleven days after challenge. It can be seen from table 1 that the minimal dose of vaccine required to produce an immunity was not determined in this experiment, but there are several conclusions which can be drawn from the data obtained. Of the 10 animals involved in the test, 6 calves demonstrated a postvaccination thermal reaction. The rise in temperature was not very high and did not persist for more than three days. Aside from this thermal reaction, all the vaccinated animals remained in good health throughout the experiment. It is shown that the calves which received 1-cc. doses of the undiluted vaccine did not demonstrate a more marked reaction to the vaccine than those which received 0.01 cc. of vaccine. It is apparent that 0.01 cc. of vaccine is sufficient to produce immunity to the challenge virus.

Since the minimal effective dose of vaccine was not determined by this experiment, various lots of the dried vaccine were tested to determine the end point. The vaccines were reconstituted to original volume with distilled water and lightly centrifuged to remove coarse particles. Dilutions were made with physiologic saline solution and administered in 1.0-cc. amounts, subcutaneously. Five vaccines which were diluted 1:1,000 produced an immunity in 2 calves and failed to do so in 3 animals. Eleven lots of vaccine out of a total of 15 produced immunity in a dilution of 1:100, whereas four lots diluted to the same extent failed to provide immunity. It became apparent during these investigations that the degree of potency and the extent to which the vaccine may be diluted varies between the vaccine lots. In general, it can be stated that most vaccines produced in the manner described previously can be depended upon to produce an immunity when diluted to 1:100. The vaccine is dried from 20-cc. amounts in 60-cc. vials so it is convenient to reconstitute the vaccine to 60 cc. Doses of less than 0.5 cc. are probably not practicable to administer to bovine animals in large numbers, so a dose of this size is recommended. Thus, the dilution recommended for administration to an animal is equal to 1.0 cc. of a dilution of 1:6, or 16 times the amount actually required to produce an immunity. This seems to be a safe margin for administration of the vaccine to large numbers of animals. Additional amounts of the reconstituted vaccine result in no ill effects, nor do they improve the degree of immunity evoked in the animal.

The reaction of individual calves to the avianized vaccine is so slight as to go almost unnoticed in most animals unless body temperatures are recorded twice daily, as is done in the case of experimental animals. An analysis of the reaction of 135 calves which were successfully immunized with various lots of vaccine reveal the figures shown in table 2.

TABLE 2—Thermal Response of Calves to the Avianized Vaccine

No. of calves vaccinated	Thermal response over 104 F.	Temp. not over 104 F.	Immune to challenge
135	103	32	135

The temperature 104 F. was selected arbitrarily as the beginning of fever in young calves. Normal temperatures, at rest, varied from 101 F. to 102.5 F. in these animals, but exercise or excitement frequently raised the temperature to 103.8 F.

The 32 calves covered in these statistics which failed to demonstrate a temperature of above 104 F. usually had at least one temperature reading above the mean. However, this peak was sometimes only 0.6 F. above the mean temperature of the animal and such a slight temperature rise might easily be caused by any number of variable conditions. It is doubtful if any significance should be placed in these small increases in body temperature. The figures indicate that approximately 76.27 per cent of the calves will demonstrate a definite temperature rise to 104 F. or higher, following administration of the avianized vaccine.

In connection with the percentage of calves which demonstrate a thermal response to the vaccine, attention is invited to the results obtained from the 10 vaccinated animals recorded in table 1. Here it may be seen that exactly one half of the animals demonstrated a rise in temperature following the vaccination. The vaccine was the same in each instance, only the dose being varied. It is interesting to note that the reactions of the calves were evenly divided between each 2 calves getting identical doses. It would appear that the thermal reaction in the case of the calves shown in table 1 was an individual reaction, not affected by the amount of vaccine administered. Further analysis of the time at which the first high temperature is observed, together with the duration of the elevated temperature, leads to the conclusion that the thermal response in calves is also probably influenced by the individual lot of vaccine being used. However, there is not sufficient data available to determine whether the reaction of individual animals or the difference between lots of vaccine is most significant in this respect.

The number of days following vaccination at which this rise in temperature occurs is illustrated in table 3:

TABLE 3—Appearance of Temperature Above 104 F. Following Vaccination

Days	3	4	5	6	7	8	9	10
Calves	5	20	33	19	17	4	2	3

The figures show that the thermal reaction may be observed most frequently from the fourth to the seventh day following vaccination. Blood drawn from vaccinated animals and transferred to normal calves during this period results in the same type of mild thermal reaction in the blood-passage calf as is observed in the vaccinated animal. Apparently, the virus in the circulating blood of the vaccinated calves on the fourth to sixth day following vaccination is of the same mild or attenuated type as that found in the mixed allantoic and amniotic fluids of eggs from advanced yolk sac passages.

The length of time the elevation of temperature persists in vaccinated animals is shown in table 4.

TABLE 4—Duration of Temperature Above 104 F.

Days	1*	2	3	4	5	6	7	8	9	10
Calves	19	13	17	22	13	9	4	2	1	3

*Represents one day or less.

It should be emphasized at this point that aside from the thermal reaction, there is little, if any, evidence of a reaction in the vaccinated animal. The animals in general remain bright and alert, eat well, and are active. Occasionally, in those animals in which a prolonged high temperature is observed, there may be a slight depression noted or a lack of appetite for a day or two. In all cases where a high temperature has persisted for periods longer than six days, some additional cause may be found aside from reaction to the vaccine.

As might possibly be expected, there is a negative correlation between the length of time after vaccination that the first high temperature is observed and the duration of the elevated temperature. This correlation is best illustrated in tabular form in table 5.

TABLE 5—Correlation Between Time of Appearance and Duration of High Temperature

From vaccination to first high temp. (days)..	3	4	5	6	7	8	9	10
Arithmetical mean of duration of high temp. (days)..	8.40	4.75	3.82	3.57	2.41	1.25	2.50*	1.00

*Represents only 2 animals—not significant.

Those animals which demonstrate a thermal reaction to the vaccine very soon after inoculation maintain a high temperature for a longer period of time than those in which the thermal response is delayed. Many different lots of vaccine were used to obtain these results and it is not known with certainty whether different degrees of potency are entirely responsible for this effect or whether it is due in part to different degrees of susceptibility of individual animals.

An attempt was made to determine whether the duration of elevated temperature in vaccinated animals or the degree of

thermal response obtained was correlated with the degree of immunity exhibited by the calves. All of the animals were immune to a subcutaneous injection of bovine-passage virus. The degree of immunity was estimated by single neutralization test of the calf serum obtained prior to challenge. Neutralization was carried out in rabbits, using a lapinized strain of rinderpest virus. The technique of this test has been described in a previous paper.[4] The result of this experiment is shown in table 6.

It can be seen from the table that all animals which demonstrated a postvaccination temperature above 105.2 F. produced sufficient neutralizing antibodies to neutralize 100 to 1,000 m.i.d.'s of lapinized virus. Animals in which the thermal response persisted for more than two days also demonstrated a high degree of neutralizing capacity. Of 9 animals which demonstrated a fever peak of less than 105.2 F., and in which the fever persisted for two days or less, there were only 4 which gave unquestionable neutralization in the concentration used, the remaining 5 animals producing a negative or questionable neutralization.

TABLE 6—Relation of the Degree and Duration of Thermal Response to Degree of Immunity

Calf No.	Vaccine lot	Highest post-vaccination temp.	Duration of high temp. (days)	Neutralization of 100 to 1,000 m.i.d.'s lapinized virus
804	C-64	103.0 F.	0	Questionable*
809	C-72	103.8 F.	0	No neutralization
801	C-61	104.0 F.	0	Questionable*
805	C-68	104.6 F.	2	No neutralization
806	C-69	104.6 F.	1	Neutralization
799	C-59	104.8 F.	2	Neutralization
803	C-63	104.8 F.	1	Neutralization
797	C-65B	105.0 F.	2	Neutralization
796	C-65A	105.2 F.	2	Questionable*
800	C-60	105.2 F.	4	Neutralization
808	C-71	105.6 F.	5	Neutralization
802	C-62	105.8 F.	4	Neutralization
798	C-58	106.6 F.	4	Neutralization
807	C-70	107.0 F.	5	Neutralization

*Questionable neutralization refers to a situation in which no temperature was observed in the test rabbit following injection of the virus-serum mixture but was immune to subsequent challenge inoculation.

It would appear from the foregoing that the degree of thermal response to the avianized vaccine as well as the duration of the elevation in temperature is, in general, correlated with the degree of immunity produced in the animal. However, thermal response to the vaccine cannot be used as the only criterion for immune response because approximately 23.8 per cent of animals which were shown to be immune on challenge failed to demonstrate a high or prolonged temperature following vaccination.

While mention has been made that 76.2 per cent of the experimental animals demonstrated postvaccination temperatures, it may be noted that all animals used were under 12 months of age, with the possible exception of 3 mature cattle. In East Africa, during the months of September and October, 1945, controlled experiments and field trials were conducted with the avianized vaccine, and in this instance only mature cattle were used. Observations made of these animals revealed that 30 per cent or less demonstrated the postvaccination febrile reactions, although all were immune on challenge.

The time at which the animal becomes immune to fully virulent bovine-passage virus following vaccination with the avianized vaccine is important from the standpoint of combating the infection in the field. In nearly all of our immunization experiments, we have held the animals fourteen days following vaccination before the challenge dose of virus is administered. This time was arbitrarily selected to take into account individual animals which might exhibit a delayed response to the vaccine. One animal to which the vaccine was administered received a challenge dose of virus on the fourth day following vaccination. The animal had a temperature of 104.6 F. on the day the challenge virus was given and remained ill for five days, after which interval recovery was prompt and no lesions were observed at autopsy. The result observed in this animal was most likely due to an interference phenomenon rather than to a true immunity. It leads to the supposition that there is no "negative" phase during immunization, at which time the animal might be more than usually susceptible to infection.

A neutralization test for serums from 10 calves which had been vaccinated with

avianized vaccine was undertaken on serums collected at various intervals following vaccination. The results of this experiment are illustrated in table 7.

TABLE 7—Appearance of Neutralizing Antibodies in the Blood of Calves Vaccinated with Avianized Vaccine

Calf No.	Vaccine Lot No.	Days after vaccination											
		3	4	5	6	7	8	9	10	11	12	13	14
674	C-40	..	..	..	..	..	†	..	†	..	†	..	†
751	C-43	..	..	..	..	..	•	..	†	..	..	..	..
747	C-48	..	..	..	..	..	•	..	•	..	BT	..	†
749	C-50	..	..	..	•	..	†	..	..	..	..	..	..
753	C-54	..	•	..	•	..	†	..	..	..	..	..	..
798	C-58	..	..	..	..	•	..	•	..	†	..	..	†
799	C-59	..	..	..	..	•	..	•	..	†	..	..	†
800	C-60	..	..	..	..	•	..	†	..	..	..	..	†
801	C-61	..	..	..	..	•	..	†	..	..	..	..	..
802	C-62	•	..	..	..	•	..	•	..	†	..	..	†

†Neutralization.
•No neutralization.
BT Broken tube.

The neutralizations were tested in rabbits against a lapinized strain of the virus. Ten to 100 m.i.d.'s of the virus were used against 1.0 cc. of calf serum in each instance.

Table 7 shows that neutralizing antibodies appear in the circulating blood of vaccinated calves in demonstrable amounts from the eighth to the eleventh day following administration of the vaccine. It is apparent that animals may be exposed to fully virulent virus eleven days after vaccination, and that fourteen days is ample time between vaccination and challenge of test animals.

The duration of immunity provided by the avianized vaccine has been investigated in 1 animal. A calf, three months of age, was vaccinated and then maintained in an isolated pen, away from contact with the disease in any form, for a period of eleven months. The serum from this animal was tested periodically for neutralizing antibodies over the entire period and was found to neutralize 10 to 100 m.i.d.'s of virus at the end of the eleventh month. The calf was challenged with 1,000 m.i.d.'s of virus eleven months after vaccination and was found to be completely immune and normal at autopsy. The result of this experiment leads to the conclusion that the immunity produced by the avianized vaccine is of considerable duration and may be sufficiently long to protect the animal for life.

Immunization tests were conducted with the avianized vaccine on a few pregnant cows to learn whether any immunity was conferred to the calf from such animals. Cow 785 was vaccinated with 0.5 cc. of avianized vaccine lot C-57, sixteen days prior to calving. Cow 816 received 0.5 cc. of lot C-52, eight days prior to calving. Inoculations were made subcutaneously and neither of the 2 animals demonstrated the typical postvaccination temperature, but both were immune when challenged with virulent bovine spleen. Cow 785 was challenged twenty-seven days after vaccination and cow 816 received the challenge dose of virus sixteen days after vaccination. They were both normal at autopsy. The calves from these 2 cows were normal in every respect. The calf from cow 785 was challenged sixty-seven days after birth and died on the fourteenth day following injection of the virus. The calf from cow 816 received a challenge dose of virus sixty-two days after birth and recovered after a severe illness of fifteen days. Blood was collected from these animals shortly after birth and at intervals during the next few days but no neutralizing antibodies could be demonstrated, using the lapinized virus and conducting the test in rabbits. Both calves were susceptible to the challenge dose and 1 recovered. No neutralizing antibodies could be shown in the colostrum of the vaccinated cows. Cow 740 was vaccinated with the avianized vaccine eight days prior to calving. She demonstrated a single, postvaccination, thermal reaction on the fourth day of 104.6 F. Unfortunately, this cow gave birth to a very weak calf which lived only fifty-seven days, and the cow developed a septic metritis and died eleven days after calving. Neither the calf nor the dam were challenged because of the intercurrent illness, but it is quite certain that the vaccine played no part in these fatalities. This cow was present at this station in 1943 and had been barren for several years. It is believed that her advanced age and previous barrenness were the principal causes of the result. It is concluded

from results obtained in these 3 cows that the vaccine may be safely administered to cows during pregnancy, and that no immunity is conferred to calves born of vaccinated animals.

Animals less than 3 months of age have not been vaccinated with the avianized vaccine but calves have been found to be susceptible to the disease at the age of 2 months. The recovery of 1 calf which became infected with rinderpest following injection of the virus, at 62 days of age, leads to the supposition that vaccination may be safely carried out at this age. Under field conditions, where a large bovine population is involved, it is recommended that animals of all ages and at any stage of pregnancy be vaccinated.

KEEPING QUALITIES OF AVIANIZED VACCINE

The milled embryos and egg fluids commonly used for vaccine production are completely inactivated and fail to provide immunity when heated to 60 C. for thirty minutes. Exposure of the fluid vaccine to room temperature (22 C.) for twenty-four hours or more reduces the potency of the vaccine, and after seventy-two hours it has been shown to be completely inactivated. The fluid vaccine held at 2 C. for ten days gradually loses potency and after fourteen days is inactive. When the vaccine is held frozen at —20 C., it is still effective after one year. Drying the vaccine from the frozen state results in improved keeping qualities at atmospheric temperatures and at 2 C. Dried vaccine containing a relatively high residual moisture (2.21 per cent) is still effective in producing immunity after fifteen months storage at 2 C. Exposure of dried vaccine having such a high residual moisture content to room temperature (22 C.) for forty-eight hours results in complete loss of potency. Vaccines dried to a residual moisture of 0.5 per cent apparently maintain their potency at 22 C. equally as well as those which are dried to 0.1 per cent moisture.

There are factors other than the residual moisture content concerned with the ability of the vaccine to maintain its potency at room temperature. Various lots of vaccine, dried under identical conditions to approximately the same degree of dryness, vary in their ability to withstand exposure to room temperature. Some lots of vaccine will maintain their potency for as long as thirty days at room temperature while others which are produced under the same conditions fail to produce immunity in calves after twenty days at the same temperature. No reason has been found for this difference in vaccine lots.

It was considered possible that the addition of protein material to the vaccine might act to protect the attenuated virus during the drying period. Fluid milk was added to one lot of vaccine for this purpose. This lot was effective after exposure to room temperature for forty-five days. Since the final product was to be dried, it seemed to be a disadvantage to add fluid milk to the vaccine, so dry, powdered skimmilk was substituted. The addition of milk results in more effective drying and production of a product which can be reconstituted in a more uniform manner. Experiments were also conducted concerning the keeping qualities of vaccines prepared from the entire egg contents rather than from the fluids and embryos alone. This type of vaccine has one great advantage over the usual vaccine. The yield of vaccine is approximately five times greater than that of the vaccine prepared in the usual manner. It was found that the vaccines prepared from the whole egg contents when freshly prepared or stored at 2 C. were equally effective as vaccines prepared from fluids and embryos alone.

Additional investigations of the keeping quality of various vaccines revealed that some lots of vaccine prepared from fluids and embryos without the addition of milk or any other substance will maintain their potency for as long as thirty days at 22 C.

An experiment designed to determine to what extent the manner of preparation of the vaccine would influence the keeping qualities of the product was carried out as follows: Three lots of avianized virus vaccine were utilized for this purpose, C-111, C-112, and C-113. From 20 to 50 eggs were

set aside from each of these lots. The entire contents of the 50 eggs were harvested and milled with dry, powdered skimmilk to the extent of 4 per cent by weight. These whole egg vaccines were designated C-111B, C-112B, and C-113B. The remaining eggs from each lot were harvested in the usual manner and, after milling, a portion of each lot was removed and milled with dry, powdered skimmilk at the rate of 4 per cent by weight. The milled fluids and embryos with milk added were designated C-111A, C-112A, and C-113A. The three lots of vaccine, made up of three different combinations, were bottled, shell frozen, dried, and packed in vacuum in the usual manner. One vial of each specimen was exposed to room temperature (22 C.) for a period of twenty days and then returned to the refrigerator. Other vials representing each variety of each of the three lots were exposed to a temperature of 22 C. for thirty days. The vaccines were reconstituted to 60 cc. with sterile phosphate buffer solution (M/10, pH 7.0), and 1.0 cc. was injected into a calf subcutaneously. After fifteen days, each animal received the usual challenge dose of virus (1.0 cc. of a 1 per cent suspension of virulent bovine spleen) subcutaneously. The results of this experiment are illustrated in table 8.

TABLE 8—Keeping Qualities of Avianized Vaccine in Various Preparations

Vaccine lot	Exposed to room temp.—20 days at 22 C.	Exposed to room temp.—30 days at 22 C.	Maintained at refrigerator temp.—2 C.
C-111	†	†	‡
C-111A	•	•	† (10^{-2})
C-111B	•	•	† (10^{-2})
C-112	†	†	‡
C-112A	†	†	• (10^{-2})
C-112B	•	•	†
C-113	•	†	‡
C-113A	†	†	‡
C-113B	†	•	†

†Immunized. •No immunity. ‡Not tested.

It can be seen from table 8 that the calf which received 1.0 cc. of the C-113 vaccine which had been exposed to room temperature for twenty days did not respond in the expected manner. The vial of the same vaccine which had been exposed to room temperature for thirty days did produce immunity in the vaccinated calf, while the one of twenty days' exposure failed. No explanation of this incongruity is apparent. Both bottles were dried on the same run in the desiccator. Aside from this obvious failure, some other findings are of doubtful nature. It would be reasonable to assume that those vaccines having the highest original titer would survive exposure to adverse temperatures to a greater degree than those with a lower titer. Lots C-111A and C-111B were both effective in a 1:100 dilution when stored at a temperature of 2 C., while vials of the same lots exposed to a temperature of 22 C. for twenty days and thirty days failed to immunize in a dilution of 1:3. On the other hand, vaccine lot C-112A which failed to immunize in a dilution of 1:100 when held in the refrigerator was effective after twenty and even thirty days at room temperature, when diluted 1:3. These results also are not what might have been expected.

Although the results of the experiment are not definite, there are a few conclusions which, in general, are probably of some value:

1) Various lots of vaccine differ in their ability to withstand adverse temperatures and this characteristic is apparently not a function of the original titer of the lot.

2) Vaccines composed of fluids and embryos apparently withstand exposure to adverse temperatures better than those containing the entire egg contents.

3) Addition of powdered milk to the embryos and fluids had no adverse effect on the keeping qualities of two out of three lots of vaccine.

4) When freshly prepared or stored at 2 C., whole egg vaccines are equally effective as the other types of vaccine.

It is concluded, therefore, that where there is a lack of suitable refrigeration between the production plant and the point of use of the vaccine, the embryos and fluids from inoculated eggs should be utilized as a vaccine. Where suitable refrigeration is available and the vaccine is required at once in large quantities, the entire egg contents may be used. The addition of skimmilk powder to the vaccine re-

sults in a product having a lower residual moisture content after drying but does not have any beneficial effect on the keeping qualities.

A total of 33 different lots of vaccine were exposed to room temperature for varying lengths of time and then tested for potency by calf inoculation. The results of these tests are shown in table 9.

TABLE 9—Summary of Results of Keeping Quality Tests

Time of exposure to 22 C.	7—10 days	10—15 days	20—30 days
Vaccines immunized....	2	6	10
Vaccines failed to immunize	2	5	8

It should be noted that the two vaccines which were not effective after exposure to room temperature for seven to ten days were lots which had been dried before the drying process had been standardized and had a relatively high residual moisture content. All of the 33 lots were effective when stored at a temperature of 2 C.

Attempts to preserve the dried vaccine by suspending in various mineral oil and beeswax preparations were not successful. Some of these preparations were effective immediately after mixing, but they did not maintain their potency when exposed to room temperature for twenty to thirty days.

Dried vaccines, packed in vacuum, in which the vacuum is not relatively high or which lose the vacuum during storage, do not retain their potency, even when kept under refrigeration. Two lots of vaccine, which were found to have a small amount or no vacuum when opened for test, failed to produce immunity after being stored at 2 C. for four months. This would indicate that the deterioration of the vaccine is probably an oxidative process. It is also indicated that those vials which fail to show a good vacuum when opened should not be used, as the results are liable to be doubtful.

Due to a difficulty in obtaining rubber stoppers for the 500-cc. bottles, a large number were dried in vacuum and stoppered in an atmosphere of nitrogen. Such lots were tested in a comparative way with other bottles from the same lot which had been dried and packed in vacuum. In the case of the two lots investigated in this manner, it was found that the nitrogen filled bottles were as equally effective as the vacuum packed bottles after exposure at 22 C. for a period of fifteen days. These findings led to the assumption that the vaccines packed in nitrogen would maintain their potency in storage as well as the vacuum packed vaccines. However, later tests indicated that the nitrogen filled bottles failed to provide immunity after nine months in storage. Since pure nitrogen is an inert gas, it must be assumed that the gas used for this purpose contained some oxygen, although tests conducted on one cylinder failed to show any oxygen on analysis.

It is interesting to note that the vaccines packed in nitrogen and those in which the vacuum was low or absent had a residual moisture content of approximately 0.7 per cent. These lots, maintained at 2 C. for four to nine months, failed to provide immunity when tested. On the other hand, one very early lot of vaccine (C-4), with a good vacuum after fifteen months in storage at 2 C., provided an excellent immune response although the moisture content was 2.2 per cent.

Results of the investigations concerning the keeping qualities of dried avianized vaccine show such marked variations between different lots of vaccine that further work in this connection is indicated. Our present knowledge of the subject may be summarized as follows:

1) Dried avianized vaccines, packed in vacuum, maintain their potency for as long as fifteen months at refrigerator temperature (2 C.), and probably longer.

2) Most lots of vaccine which have been properly dried and stoppered under vacuum can be depended upon to be effective after seven to ten days' exposure to a temperature of 22 C.

3) Several lots of vaccine have been found to be effective after exposure to a temperature of 22 C. for twenty days, thirty days, and even forty-five days.

4) Reconstituted from the dried state, the vaccines are completely inactive after

forty-eight hours at 2 C. It is recommended that no reconstituted vaccine be used after a twelve-hour period.

5) Vaccines packed in an atmosphere of nitrogen do not maintain their potency in storage and those packed in vacuum are of doubtful potency if the vacuum is low or completely lost.

POTENCY TESTS OF AVIANIZED VACCINE

It has been noted in a previous paper[1] that the most positive means of determining the potency of the avianized vaccine is by the subcutaneous injection of calves with measured amounts or various dilutions of the reconstituted dried material. The calves are challenged with 100 to 1,000 m.i.d.'s of virus after fourteen days from the date of vaccination.

Adaptation of the rinderpest virus to rabbits, reported previously,[4] provided a means of testing the neutralizing capacity of serums from immune animals. Later it was shown that chicks would produce neutralizing antibodies against rinderpest virus when the avianized vaccine was administered intravenously.[5] By the use of day-old chicks for the production of antibodies and carrying out the neutralization test in rabbits, it was possible to determine the potency of the avianized vaccine without the use of calves. There are several disadvantages to the routine use of this method of determining the potency of different lots of vaccine, chief of which are the number of manipulations necessary and the length of time required to complete the test. Also, it is not uncommon to encounter a rabbit which is refractory to the virus and does not respond in the proper manner.

Injections of the reconstituted, but undiluted, vaccine in rabbits intravenously results in the production of immunity to the lapinized virus, provided the dose is sufficiently large. The period required for development of immunity is twenty-one days and the rabbits are challenged with ten to 100 m.i.d.'s of lapinized virus at that interval, following administration of the vaccine. Doses of 1.0 cc. or 2.0 cc. produced immunity in approximately 57 per cent of the rabbits tested. When the dose was increased to 3.0 cc. of undiluted vaccine, about 70 per cent of the rabbits were immunized. When 5.0 cc. of the vaccine was used, only two vaccines out of ten failed to immunize rabbits. None of the vaccines which failed to provide immunity in calves were effective in producing immunity in rabbits. Thus, this method of testing vaccines for potency might be estimated to be approximately 80 per cent effective. In locations where susceptible cattle are not readily available for testing vaccines, this method might find some use, especially if those vaccines which failed to provide immunity in rabbits could be retested in calves.

A method for demonstrating the presence of virus in egg fluids by the complement fixation test will be described in a later paper. By this means, presence of the virus can be determined prior to mixing the fluids with the milled embryos and before freezing and drying.

Thus far, we have not been able to devise a means of testing the potency of dried avianized vaccine which is completely as satisfactory as when the vaccine is tested in calves.

SUMMARY

An attenuated rinderpest vaccine, produced from cultures of virus in embryonating eggs, has been shown to produce a solid and lasting immunity in cattle. The postvaccination reaction produced is mild and of short duration. The immune response is prompt, most animals demonstrating neutralizing antibodies within ten days. Animals of any age and in any period of pregnancy may be safely vaccinated, but no immunity is conferred to the calves from cows vaccinated during the pregnancy. The dried vaccine, packed in vacuum, will maintain its potency for as long as fifteen months, when stored at a temperature of 2 to 5 C. The dried vaccine will not remain potent for long periods at high atmospheric temperatures. It rapidly loses its ability to immunize after being reconstituted from the dry state and should be administered within twelve hours. The only

completely satisfactory method of determining the potency of the dried vaccine is by administration of the vaccine to susceptible cattle and challenging these animals with fully virulent material at the end of a fourteen-day period.

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Rinderpest

XV. Morphological Changes in the Blood of Young Cattle During Rinderpest and After Vaccination with Attenuated Virus Vaccine

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A STUDY of the cellular elements of the blood of young bovine animals during the course of rinderpest was undertaken with a view to finding some characteristic which would be an aid in diagnosis of the disease. Evidence of the presence of rinderpest is quite plain in those countries where the disease is known to exist in enzoötic proportions, but immature bovine animals under experimental conditions frequently die before the classic clinical symptoms develop. Sometimes there are no characteristic lesions at autopsy, which will distinguish the disease from many other ailments to which bovine animals are incident.

Hutyra and Marek[1] quote Baldry in stating: "[There is] an increase in leucocytes from the onset of fever till the fifth day of the disease, after which their number in unfavorable cases decreases to 4,000 per cmm. If the animal survives, they increase in number to 28,000 to 30,000, after which follows a rapid decrease till the normal condition is established on the twentieth day." Presumably, these findings are from adult bovine animals and may include some suffering from complicating or intercurrent conditions. In any event, these findings do not agree with those obtained at this station where the disease was observed under carefully controlled, experimental conditions.

Other workers with virus diseases of animals have in some instances reported a marked leucopenia. Dinwiddie[2] first drew attention to the leucopenia which characterizes hog cholera. His findings were confirmed by Lewis and Shope[3] and subsequent workers. Kernkamp[4] made an intensive study of the blood picture in hog cholera and his findings also included a marked leucopenia. Hammon and Enders[5] demonstrated the occurrence of leucopenia in feline enteritis. Kirk and Collins[6] have called attention to the leucopenia associated with a rise in temperature in ferrets occurring prior to visible clinical symptoms of distemper. Leucopenia also has been listed as a symptom of many human viral and rickettsial diseases such as sandfly fever, dengue, Rift Valley fever, and Rocky Mountain spotted fever.

In the present work, young bovine animals (3 months to 1 year) of both sexes and of mixed breeds were used for the reason that they were better adapted to small isolation units than adult cattle. The animals were purchased locally and maintained in a quarantine stable until they were moved to the isolation units[7] for experimental inoculation. The blood was collected from the jugular vein, in 5.0-cc. amounts, in tubes containing 10.0 mg. of dry potassium oxalate. Blood was drawn at the same time each morning—prior to feeding. The white cell and erythrocyte counts were made within one hour of drawing the blood, and slides for differential white cell determinations were made immediately after collection. For the differential counts of neutrophiles classification was limited to "new" and "old" forms; those with segmented nuclei being counted as "old" and all others "new."

In order to obtain the normal blood picture of these young bovine animals, 42 counts were made on 21 animals. The mean total counts per cubic millimeter were as follows: white cells 7,300 and erythrocytes 8,100,000. The mean percentages of the various types of cells were: lymphocytes 70;

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neutrophiles 21.2 (16 "old" and 5.2 "new"); eosinophiles 5.4; monocytes 2.4; basophiles 1.0. The hematocrit (Wintrobe) averaged 55 per cent and hemoglobin (Tallqvist) 85 per cent.

Preliminary determinations made on available animals in various stages of rinderpest indicated little change in the erythrocyte count, hematocrit or hemoglobin estimations, but definite deviations from the normal white cell counts were observed. There probably is a slight decrease in erythrocytes at the height of the disease but the change is not as striking or as significant as that noted in the white cells. Another observation made, but not investigated, shows that in the very late stages of the disease just prior to death, the blood becomes thick and dark colored, does not clot, and the serum does not separate.

In this study, only the white cells were considered because their deviations from the normal were the most significant. Further, previous workers have demonstrated that the virus in the circulating blood is contained in, or attached closely to, the white cells. Daily counts were made on 3 calves starting on the day the animals received a challenge dose of rinderpest virus. The injection consisted of 1.0 cc. of a 1 per cent suspension of finely ground spleen from a calf destroyed at the stage of the disease when the virus is in greatest concentration in that organ, *i.e.*, thirty-six to forty-eight hours after the initial rise in temperature. The amount administered was equivalent to at least 100 m.i.d.'s (minimal infective doses). Temperatures of the animals were recorded twice daily in order to compare the status of the disease with the cell counts. Table 1 shows the results obtained in the 3 calves.

Temperatures recorded in the table are those obtained in the morning at the time the blood was drawn. Clinically, all 3 animals developed anorexia on the day following the first high temperature reading, and diarrhea appeared three to five days following the initial rise in temperature. The anorexia persisted for eight to eleven days and the diarrhea from seven to fourteen days. Following the onset of diarrhea, all animals became weakened and lost weight rapidly. Surprisingly, all 3 animals recovered from the disease but in each case the convalescent period was prolonged.

The table illustrates the sudden, pronounced, and prolonged decrease in white cell numbers beginning at approximately the same time that a significant temperature rise is observed in the animals. A distinct diphasic fluctuation of the white cell count is noted in each case, with a tendency to increase following the initial decrease followed by another drop in numbers. The white cell count never approaches normal

TABLE I—White Cell and Thermal Response to Rinderpest Virus

	CALF 975		CALF 976		CALF 977	
Days	White cells	Temp. (F.)	White cells	Temp. (F.)	White cells	Temp. (F.)
1	6,100	101.8	8,900	101.4	8,750	101.6
2	7,500	101.4	8,700	101.4	9,400	100.8
3	6,000	101.0	7,500	102.4	4,800	101.4
4	3,900	106.2	3,150	106.2	2,500	105.4
5	1,000	105.6	1,600	105.0	1,400	104.0
6	1,200	105.6	1,900	105.4	1,900	104.0
7	2,750	104.6	4,500	106.4	2,400	104.0
8	2,400	104.4	5,500	105.8	2,350	104.6
9	1,350	102.4	6,000	105.8	1,450	104.0
10	1,350	104.0	4,150	102.6	3,300	102.8
11	900	101.6	3,450	102.4	2,250	102.4
12	1,300	101.2	2,800	103.0	1,750	101.6
13	900	100.2	2,250	102.6	2,450	101.6
14	1,450	100.6	2,900	102.2	3,000	101.6
15	1,250	101.6	2,950	101.2	3,300	101.6
16	1,600	100.4	3,950	101.6	2,450	101.2
17	2,800	101.4	4,550	101.8	3,750	101.8
18	3,000	100.4	4,600	101.8	3,400	101.6
19	3,500	101.2	6,000	101.6	3,600	100.0
20	3,400	100.4	4,600	101.6	3,650	100.2
21	4,700	100.6	5,200	102.0	3,150	100.8
22	5,200	100.8	5,600	101.8	4,500	101.0
23	4,200	100.4	4,600	102.0	4,000	101.0
24	3,550	100.6	5,400	102.3	5,500	101.2

figures during the course of the uncomplicated disease. Normal white cell counts are not observed in recovered cases until approximately five weeks after clinical symptoms subside.

Chart 1 illustrates graphically the findings on 1 calf as typical of the disease in such animals. This calf was a grade Ayrshire male, 7 months of age at the time of inoculation.

The Schilling index on the chart does not illustrate the extreme and sudden "shift to the left" in neutrophiles as it actually appears in the daily counts. In order to point out this change, as well as the relative increase in neutrophiles, table 2 has been prepared. It should be noted that the increase in neutrophiles is only relative and that there is an actual decrease as shown by table 1 and chart 1.

TABLE 2—Differential Neutrophile Response to Rinderpest Virus

Days	CALF 975			CALF 976			CALF 977		
	New forms	Old forms	Total	New forms	Old forms	Total	New forms	Old forms	Total
1	6	17	23	10.5	14.5	25	5	29	34
2	3	23	26	14.5	14.5	29	3	35	38
3	6	18	24	24.5	10	34.5	5	31	36
4	16	41	57	12.5	8.5	21	14	35	49
5	4	18	22	16	5.5	21.5	5	28	33
6	32	18	50	26.5	9.5	36	22	17	39
7	42	17	59	31	6.5	37.5	17	25	42
8	55	15	70	59.5	3.5	63	28	21	49
9	45	11	56	71.5	3	74.5	18	30	48
10	26	8	34	52	18	70	11	29	40
11	38	5	43	58	15	73	20	14	34
12	20	6	26	41	32	73	6	26	32
13	30	8	38	33	25	58	4.5	14	18.5
14	19	17	36	9	27	36	1	14.5	15.5
15	13	15	28	7	30	37	6	25.5	31.5
16	13	13	26	19	29	48	6	7	13
17	8.5	23.5	32	14	22	36	2	7	9
18	7	30	37	6	18	24	1	14	15
19	3.5	27.5	31	5	25	30	1.5	15.5	17
20	14	22.5	36.5	7	26	33	5.5	5.5	11
21	5	24	29	2	23	25	3	11	14
22	6	36	42	2	20	22	3	9	12
23	8.5	26	34.5	1	19	20	3.5	16	19.5

During studies concerning immunization with avianized rinderpest virus vaccine,[8] it was noted that there was occasionally a slight rise in temperature following injection of the material. This observation led to an investigation of the cellular elements of the blood following vaccination.

The vaccine used in this experiment was prepared from the milled embryos and mixed allantoic and amniotic fluids from eggs which had been inoculated with an attenuated strain of rinderpest virus.[9] Six different lots of vaccine were used, four of which were presumed to be potent and the remaining two lots of doubtful potency. The calves used in the test were maintained in a large isolation pen. Temperatures of the animals were recorded twice daily. Blood was obtained and white cell counts were made in the manner described previously. All animals received a challenge injection of virus equivalent to 100 m.i.d.'s fifteen days after vaccination. The results obtained are shown in table 3.

It may be seen that there was, in general, a reduction in the white cell count on the fifth to the ninth day after vaccination. The change is not regular in occurrence or degree but differs greatly between individual calves. The animals which received the vaccines of doubtful potency (1,015 and 1,017) were shown to be susceptible to the disease when challenged, and the effect of the virus on the cell count of these animals can be seen in the postchallenge period. The fact that there was a decrease in white cell numbers in these 2 animals leads to the supposition that the vaccines administered to them were not completely inactivated. It is presumed that some attenuated virus was present in these two lots of vaccine but that it was not sufficiently potent to produce an immune response in the calves.

Chart 2 illustrates graphically the changes observed in the blood of calf 1,013 following administration of the avianized

Chart I

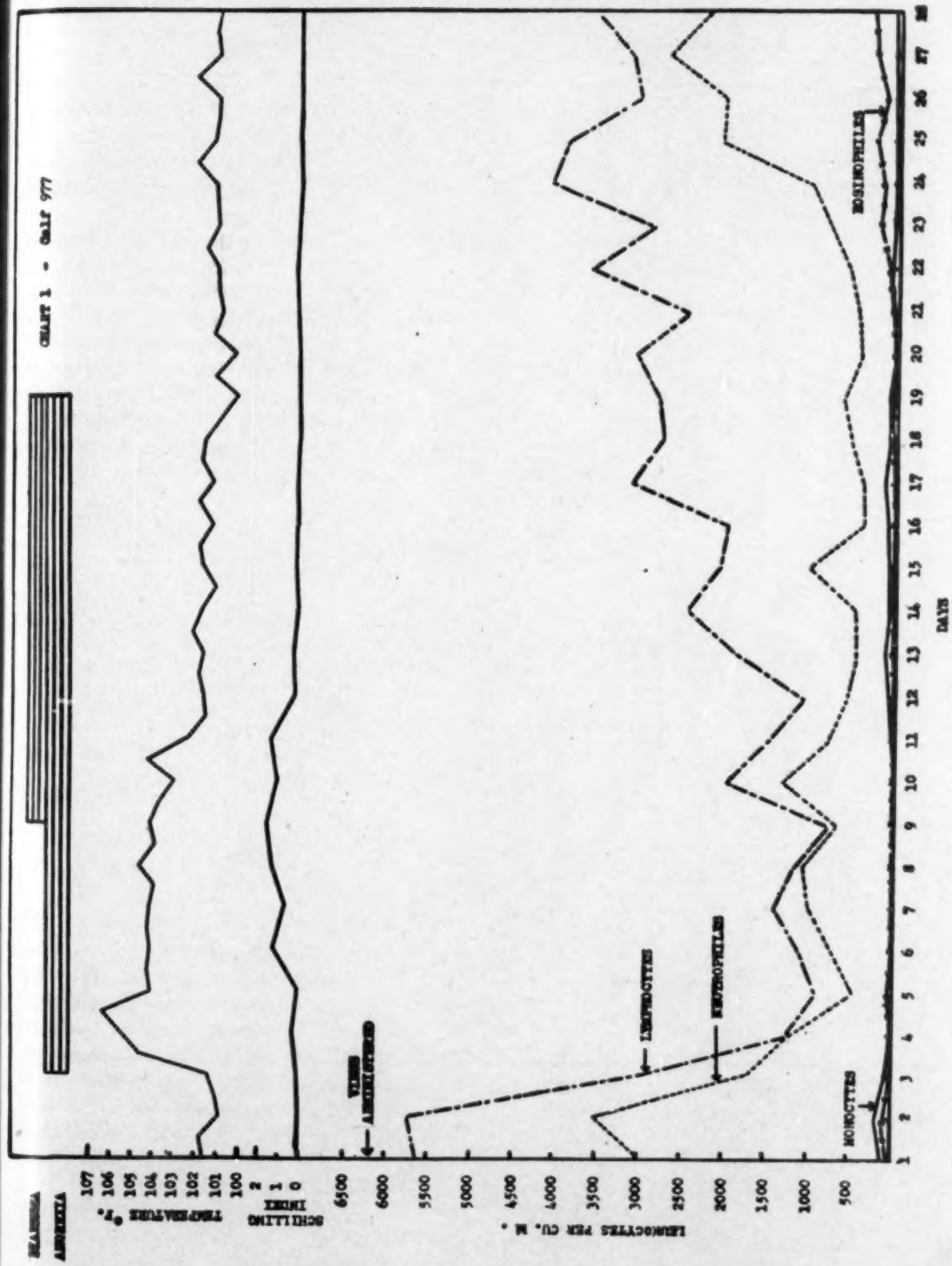
CHART 1 - Calf 977
TEMPERATURE °F.
OSCILLATING INDEX
LEUCOCYTES PER CU. M.
DAYS
EOSINOPHILES
LYMPHOCYTES
NEUTROPHILES
MONOCYTES

Chart 2

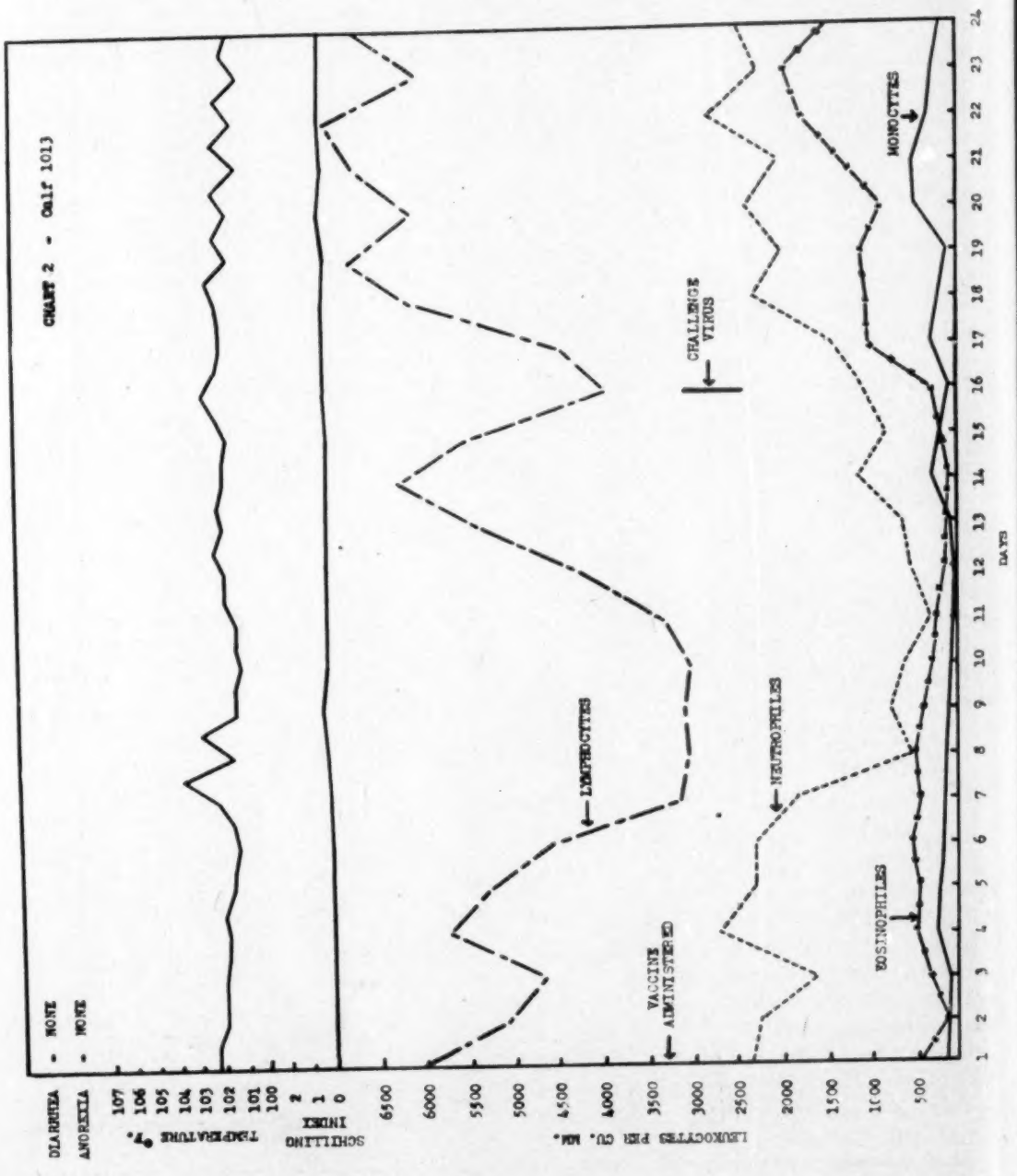
CHART 2 - Calf 1013
- NONE
- NONE
DIARRHEA
ANOREXIA
TEMPERATURE °F.
SCHILLING INDEX
LEUKOCYTES PER CU. MM.
DAYS
VACCINE ADMINISTERED
CHALLENGE VIRUS
LYMPHOCYTES
NEUTROPHILES
EOSINOPHILES
MONOCYTES

vaccine and subsequent challenge with virus. A slight shift to the left in neutrophiles was observed about nine days after vaccination and again about four days following challenge. Nearly all the animals experienced this slight increase in new forms of neutrophiles following vaccination, but the shift was only slight compared to that seen in animals which had received only the virus.

TABLE 3—White Cell Counts Following Vaccination with Avianized Rinderpest Vaccine

Days		CALF NUMBER					
		1011	1012	1013	1015	1016	1017
1	Postvaccination Period	9,450	9,800	9,050	9,550	7,350	9,750
2		7,550	9,100	7,650	14,750	6,950	9,050
3		9,200	10,850	6,800	10,200	9,700	9,550
4		8,550	10,750	9,200	9,250	7,200	8,450
5		8,150	9,800	8,400	9,450	7,350	8,150
6		8,300	8,350	7,800	8,850	5,700	9,950
7		8,900	7,250	5,700	7,350	5,050	9,350
8		7,750	9,150	4,350	3,600	5,300	4,700
9		5,000	5,300	4,500	5,700	5,350	9,200
10		4,700	6,350	4,200	5,750	5,700	8,100
11		5,050	13,050	3,950	5,850	5,550	7,600
12		5,750	9,450	6,450	6,000	4,700	5,550
13		6,350	9,850	6,050	7,250	6,400	9,500
14		5,000	11,300	7,850	9,850	6,650	8,700
15		5,050	11,350	6,700	8,550	7,600	8,300
16		6,750	9,750	5,550	8,450	7,800	5,950
Average		6,968	9,465	6,512	8,150	6,522	8,240
17	Postchallenge Period	5,350	6,550	7,350	8,300	8,650	4,650
18		5,650	7,350	9,700	8,350	8,150	7,200
19		8,450	8,950	10,100	8,000	7,350	7,300
20		10,700	9,700	9,950	6,350	9,900	9,650
21		8,850	9,000	11,400	3,050	7,400	5,250
22		9,750	11,850	13,250	3,400	6,300	4,450
23		7,650	10,300	9,700	2,750	9,200	4,850
24		9,050	11,800	10,150	3,650	9,050	4,700
Average		8,181	9,437	10,200	5,481	8,500	6,006
Result		Immune	Immune	Immune	Not Immune	Immune	Not Immune

SUMMARY

Uncomplicated rinderpest in young bovine animals is characterized by a marked leucopenia beginning on the third or fourth day after injection of the virus and persisting for several weeks in those animals which survive. The decrease in white cells appears at approximately the same time that there is a rise in body temperature and prior to the appearance of visible clinical symptoms. There also appears a marked shift to the left in neutrophiles, following the sudden decrease in numbers.

Administration of avianized rinderpest vaccine to young bovine animals resulted in a slight and transitory decrease in white cells on the fifth to the ninth day following injection. This slight decrease in white cells, with prompt return to normal numbers, is believed to be due to the activity of the attenuated virus.

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Rinderpest

XVI. Complement Fixation Test for Rinderpest

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REPORTS indicate that the c.f. (complement fixation) test has been used successfully in the diagnosis of several virus diseases, by Levens and Enders,[1] and Habel[2] in mumps, eastern and western encephalomyelitis, St. Louis encephalitis, and others by Casals,[3,4] and Howitt.[5] To date, no satisfactory c.f. test has been reported for rinderpest. Several attempts to utilize the c.f., precipitation, and flocculation tests have been reported by a number of investigators. Angeloff, Schein, and Mrowka[6] revealed only negative results. Ruppert[6] claimed to have success with the precipitation test, using organ extracts prepared with chloroform and physiologic saline as an antigen. Nakamura,[6, 7] Sasaki and Nasaki[6] reported successful use of the c.f. test with extracts of various organs, especially lymph glands, and coctoantigens. Daubney[6] claimed positive results using the flocculation test with an antigen prepared from alcoholic tissue extracts. Nakamura[6, 7] also claimed to be able to demonstrate the existence of different strains of the virus by means of the c.f. test. These results have not been confirmed, and it is doubtful whether the methods employed are practical for the diagnosis of the disease. Shope[8] reported favorable results with the c.f. test for rinderpest, using an antigen prepared from infected calf spleen, but serum titers were too low to yield dependable results.

The c.f. test reported herewith was principally for the purpose of demonstrating the multiplication of the virus in the allantoic and amniotic fluids of inoculated embryonating eggs. Egg fluids are used at this station for seed virus to produce an attenuated avianized vaccine. While working on this problem a satisfactory antigen was found which did not require any special processing. The AA (mixed allantoic and amniotic) fluids from eleven-day infected eggs produced a suitable antigen with no anticomplementary tendencies. A similar method has been reported for the virus of mumps.[1, 2] The virus of rinderpest does not produce macroscopic lesions in embryonating eggs. Consequently, it was necessary to test the virus content of the AA fluids for potency in susceptible calves before the fluids could be utilized safely for seed.

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PREPARATION OF ANTIGEN

The antigen consists of AA fluids harvested from infected eggs. Seven-day embryonating eggs are inoculated *via* the yolk sac with 0.5 cc. of avianized seed virus. After ninety-six hours' incubation, the fluids are harvested aseptically from all eggs containing living embryos, and pooled. It is desirable that 60 or more eggs be used for each lot. The fluids are distributed in 20-cc. amounts in 60-cc. vaccine bottles and placed in the freezer at —20 C. In this way, the antigenicity can be maintained for long periods and any bacterial activity inhibited.

Other portions of the egg have been used as antigens with variable results. Yolk sac fluids, amniotic, allantoic, and yolk sac membranes in various dilutions have yielded positive reactions. Yolk sac membranes ground and suspended in physiologic saline solution are anticomplementary in low dilutions. The substitution of normal AA fluids from 11-day embryos as a diluent aids in overcoming this tendency, since egg fluids usually stimulate complement activity.

COMPLEMENT TITRATIONS

The first consideration regarding the titration of AA fluids is to check the hemolytic activity in the presence of complement.[9] Chart 1 outlines the protocol followed in the titrations employed at this station.

The various series of AA fluids from 11-day eggs appeared to enhance the hemolytic activity of the complement, some quite markedly. This property is found in both normal and infected egg fluids. Several inactivated bovine serums were also found to enhance the complement activity and were responsible for the "zoning phenomenon" in serum titrations. The complement titrations have also been run in the presence of antigen plus positive serum, which enhanced the complement. This procedure was found advisable in determining the strength of the complement when both the serum and antigen showed enhancing qualities. In the c.f. reaction, using viral antigens, the fixation of the complement requires several hours as compared with the c.f. with bacterial antigens. Chart 3 shows the length of time required for the fixation of complement by antigen and antibody.

CHART I—Complement Titrations

Amount of complement (diluted 1:30)	.10	.15	.20	.25	.30	.35	.40	.45	.50	Control
Amount of antigen	.50	.50	.50	.50	.50	.50	.50	.50	.50	.50
Amount of inactivated serum	.10	.10	.10	.10	.10	.10	.10	.10	.10	.10
Incubate in water bath at 37 C. for 30 min.										
.5 cc. 2% sheep cells + .5 cc. (hemolytic amboceptor containing 2 u.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Incubate in water bath at 37 C. for 30 min.										
Complement only	4+	4+	3+	2+	+	—	—	—	—	4+
Complement + normal allantoic and amniotic egg fluids	4+	2+	+	—	—	—	—	—	—	4+
Complement + infected allantoic and amniotic egg fluids	4±	2+	+	—	—	—	—	—	—	4+
Complement + egg fluid antigen + serum calf 971	3+	+	—	—	—	—	—	—	—	4+
Complement + egg fluid antigen + serum calf 1,001	4±	2+	+	—	—	—	—	—	—	4+
Complement + serum calf 515	2+	—	—	—	—	—	—	—	—	4+
Complement + serum calf 971	3+	+	—	—	—	—	—	—	—	4+
Complement + serum calf 1,001	4+	3+	+	—	—	—	—	—	—	4+
Complement + dehydrated antigen lot 1	4+	4+	3+	2+	+	±	—	—	—	4+

u. = units.

ANTIGEN TITRATIONS

Following the titration of the complement in the presence of pooled AA fluids, the test used is here outlined. The test is set up using twofold dilutions of the AA fluid antigen. The amount of antigen per tube varies from 0.1 to 2.5 cc.; the amount of inactivated positive or negative bovine serum 0.1 cc. per tube and the amount of complement is two units contained in 1.0 cc. Controls include one for the antigen and one for the positive serum. Normal AA fluids from 11-day eggs have also been used as controls in the presence of positive and negative serum with negative results. The test is then placed in the refrigerator for eighteen to twenty hours at 4 to 7 C. If the antigen is free from bacterial contamination, good results may be obtained by leaving the test at room temperature overnight, or for four hours at room temperature and two hours in the water bath at 37 C. (chart 3). The presence of bacteria makes the two latter procedures objectionable since the complement is destroyed by bacteria. The hemolytic system is added, using two units of hemolysin in a volume of 0.5 cc. and 0.5 cc. of a 2 per cent suspension of sheep cells. The test is incubated for thirty minutes at 37 C. and then allowed to stand at room temperature for one or two hours before reading. The degree of hemolysis is recorded as (—) negative, +, 2+, 3+ and

CHART 2—Antigen Titrations

Antigen	Serums 0.1 cc.	Antigen Dilutions .1	.2	.3	.4	.5	.6	.7	.8	.9	1.0	1.5	2.0	2.5
	Neg.	—	—	—	—	—	—	—	—	—	—			
27-27D—Allantoic & amniotic fluids.	Pos.	2+	3+	4+	4+	4+	4+	4+	4+	4+	4+			
	Neg.	—	—	—	—	—	—	—	—	—	—			
27-27D—Amniotic fluids	Pos.	3+	4+	4+	4+	4+	4+	4+	4+	4+	4±			
	Neg.	—	—	—	—	—	—	—	—	—	—	—	—	—
27-27D—Allantoic fluids	Pos.	—	—	—	—	—	—	+	2+	2+	3+	4+	4+	4+
	Neg.	—	—	—	—	—	—	—	—	—	—			
27-27E—Allantoic & amniotic fluids.	Pos.	—	—	+	2+	2+	3+	4+	4+	4+	4+			
	Neg.	—	—	—	—	—	—	—	—	—	—			
27-27E—Amniotic fluids	Pos.	—	+	3+	4+	4+	4+	4+	4+	4+	4+			
	Neg.	—	—	—	—	—	—	—	—	—	—	—	—	
27-27E—Allantoic fluids	Pos.	—	—	—	—	—	—	—	—	+	2+	3+	4+	
	Neg.	—	—	—	—	—								
27-27E—1:100 dilution of amniotic membrane	Pos.	2+	3+	4+	4+	4+								
	Neg.	—	—	—	—	—								
27-27E—1:50 dilution of allantoic membrane	Pos.	—	+	2+	3+	4+								
	Neg.	—	—	—	—	—								
27-27E—1:50 dilution of yolk sac membrane	Pos.	—	2+	3+	4+	4+								
	Neg.	—	—	—	—	—	—	—	—	—	—			
27-27E—undiluted yolk fluids.....	Pos.	—	—	—	—	—	—	—	2+	3+	4+			
	Neg.	—	—	—	—	—	—	—	—	—	—	—	—	—
RL14CA7YS17—fluids	Pos.	—	—	—	—	—	—	—	—	—	—	—	—	+
	Neg.	—	—	—	—	—	—	—	—	—	—	—	—	—
RL14CA7YS18—fluids	Pos.	—	—	—	—	—	—	—	—	—	—	±	2+	3+
	Neg.	—	—	—	—	—	—	—	—	—	—	—	—	—
RL14CA7YS19—fluids	Pos.	—	—	—	—	±	+	2±	2+	3±	3+	4+	4+	4+
	Neg.	—	—	—	—	—	—	—	—	—	—	—	—	—
RL14CA7YS20—fluids	Pos.	—	—	—	—	—	—	—	—	—	±	2+	3+	4+
	Neg.	—	—	—	—	—	—	—	—	—	—	—	—	—
RL14CA7YS22—fluids	Pos.	—	—	—	—	+	2+	3±	3+	4±	4+	4+	4+	4+
	Neg.	—	—	—	—	—								
RL14CA7YS22—1:50 dilution amniotic membrane	Pos.	2+	3+	4+	4+	4+								
	Neg.	—	—	—	—	—								
R18OYSF9—1:50 dilution amniotic membrane	Pos.	—	+	2+	3+	4+								
	Neg.	—	—	—	—	—	—	—	—	—	—			
Heated allantoic & amniotic fluids 1 hr. at 60 C.....	Pos.	—	—	—	+	2+	2+	3+	3+	4+	4+			
	Neg.	—	—	—	—	—	—	—	—	—	—			
Unheated allantoic & amniotic fluids	Pos.	—	—	+	2+	3+	4+	4+	4+	4+	4+			
	Neg.							—	—	—	—	—	—	
Desiccated allantoic & amniotic fluids—antigen 1	Pos.							+	2+	3+	3+	4+	4+	
	Neg.							—	—	—	—	—	—	
Dessicated allantoic & amniotic fluids—antigen 2	Pos.							—	—	—	+	2+	3+	
	Neg.				—	—	—	—	—					
Allantoic & amniotic fluids Fresh antigen lot 1.....	Pos.				2+	3+	4+	4+	4+					
	Neg.				—	—	—	—	—					
Allantoic & amniotic fluids Fresh antigen lot 2.....	Pos.				—	+	2+	3+	4+					
Allantoic & amniotic fluids	Neg.	—	—	—	—	—	—	—	—	—	—			
Fresh antigen lot 4.....	Pos.	±	+	2+	3+	4+	4+	4+	4+	4+	4+			

4+, the latter representing complete fixation.

For the purpose of titrating serums with AA fluids as an antigen, the first tube in the antigen titration to exhibit a 4+ reaction is considered as one unit. All the AA fluids used as antigens have shown a 4+ reaction at 0.3 to 0.5 cc. Consequently, in all the serum titrations noted in this report, the dose of the antigen was 1.0 cc. representing two units. Results indicate that a slight excess of antigen is desirable for dependable reactions.

CHART 3—Effect of Time and Temperature During Incubation Period for Complement Fixation Test

Antigen + Antibody + Complement				
Refrigerator 4-7 C.	Room temp.	Water bath 37 C.	Hemolytic system	Result
6 hr.	....	....	Hemolytic system added after incubation period.	Complete hemolysis
....	2 hr.	....		Complete hemolysis
....	2 hr.	2 hr.		Complete hemolysis
....	4 hr.	2 hr.	Placed in water bath at 37 C. for 30 min.	No hemolysis
....	12-15 hr.	....		No hemolysis
18-20 hr.	....	....		No hemolysis

Chart 2 illustrates some typical titration reactions. The AA fluids of series 27-27D had a higher titer (1 : 5) than most of the seed fluids tested. This series had 27 passages on the chorioallantoic membrane and 27 in the yolk sac, a total of 54 passages. The amniotic fluid from this particular series had a titer of 1:10, while the allantoic fluid had a titer of 1:1. In the 27-27E series, the results obtained with the AA fluids were similar to the D series. The titer of the amniotic membrane was 1:500; allantoic membrane 1:125; yolk sac membrane 1:366, and the yolk sac fluid 1:1.

In another series, commencing with RL14CA7YS17, the antigen appeared first in the fluids in demonstrable amounts at the 24th total passage level as shown on chart 2, with a gradual increase to the 26th passage level. At the 27th passage, a decrease in antigenicity was noted. By the 29th passage level, the fluids showed a definite increase to a titer of 1:1.2. The amniotic membrane had a titer of 1:220.

In a further series—R18OYSF9—which had no chorioallantoic membrane passages, the fluids first showed a positive titer on the seventh yolk sac passage. The amniotic membrane had a titer of 1:165. The reasons for the variations in the early egg series are difficult to explain. Therefore, the ability to check the multiplication of the virus in the embryonating eggs is of considerable value.

In testing various components of infected eggs, the highest concentration was found in the amniotic membranes. Titers varying from 1:100 to 1:500 were obtained from various groups of eggs. A similar finding is reported in eggs infected with the virus of mumps.[1] The combined AA fluids have not exhibited a titer of over 1:10, usually from 1:2 to 1:5.

It was found that viral antigens may be inactivated by heat at 60 C. for one to two hours and still retain their antigenicity (chart 2). The titer is reduced slightly but if the antigen has a high titer before heating, it may be used. A number of serum titrations have been carried out using heated antigen with satisfactory results. The use of heat-inactivated antigens for neurotropic viruses has been reported by Casals.[10] Antigens inactivated with formalin were not satisfactory due to anticomplementary reactions. Similar results were obtained by Casals.[11] Desiccation of egg fluid antigens from the frozen state reduced the antigenicity and also caused them to become slightly anticomplementary as compared to fresh fluids. From the results obtained, it would appear that if the antigen had a sufficiently high titer it could satisfactorily be dehydrated[10] (chart 3).

At attempt was made to show the relationship between the *in vitro* and *in vivo* titer of egg fluids. Infective AA fluids with an *in vitro* titer of 1:2 produced a complete immunity in a calf with a 10^{-2} dilution. An injection of a 10^{-3} dilution of AA fluids of the same *in vitro* titer produced no immune response.

In harvesting allantoic and amniotic fluids for seed, an occasional contamination

occurred. Filtration of the fluids was attempted using the Seitz (EK) pad, Jenkins, and Berkefeld (W and N) filters. Subsequent titrations revealed that the antigenic factor was removed during the process of filtration.

COMPLEMENT FIXATION OF BOVINE SERUMS

Chart 4 illustrates the protocol used in titrating bovine serums. To date, over 200 bovine serums have been checked by the c.f. test. The majority of these serums were obtained from calves vaccinated with a chloroform-inactivated bovine tissue vaccine or with the attenuated avianized vaccine. The serums had been collected over a period of twenty months. Nearly all of them had been previously checked by the neutralization test.[12] Most of the serums were collected just prior to challenge. All animals were challenged with potent virus ten to seventeen days following vaccination to determine immunity. Out of 170 postvaccination serums, 36 were negative and, of these, four gave pseudopositive reactions. Of 127 calves vaccinated with avianized vaccine, 32 serums were negative to the c.f. and neutralization tests. These were nonimmune on challenge with the live virus. Three serums gave suspicious reactions and one was completely negative to the c.f. test, but all were positive to the neutralization test and the vaccinated animals were immune to challenge. Thus, of 95 animals which had been vaccinated with the avianized vaccine and were subsequently immune to challenge, 91 were shown to have complement-fixing antibodies. The 3 animals which gave suspicious reactions and the 1 negative to the c.f. test may be accounted for by the slow development of complement-fixing antibodies (chart 5).

The animals which were vaccinated with the bovine tissue vaccine gave the following results: Out of 43 calves, 4 were definitely nonimmune on challenge and were negative to the c.f. and neutralization tests. Three gave suspicious reactions to the c.f. test but were positive to the neutralization test and proved immune on challenge. Five were considered nonimmune but exhibited a marked c.f. reaction; 3 of the 5 were positive to the neutralization test (the other 2 were not checked). When challenged with 1,000 to 10,000 m.i.d.'s of virus, these 5 animals were mildly to moderately ill, and recovered. According to the standard for bovine tissue vaccine established at this station, any animal which, subsequent to challenge with 1,000 to 10,000 m.i.d.'s of virus, following vaccination, revealed a febrile reaction was considered nonimmune. Out of 39 calves which survived a challenge with the live virus, 36 gave satisfactory c.f. reactions.

Chart 4 demonstrates the typical reactions encountered in testing 170 bovine serums from animals vaccinated with the two types of vaccine against rinderpest.

Five bovine serums were heated to 60 C. for sixty minutes and dried from the frozen state in a mechanically refrigerated desiccator.[13] When reconstituted, they still retained their complement fixing activity with little loss of titer. Using 1.0 cc. of serum as a basis for determining its titer, most postvaccination serums had titers ranging from 1:6 to 1:500. Three animals which recovered from inoculations with the live virus had serum titers of 1:500.

DEVELOPMENT OF COMPLEMENT FIXING ANTIBODIES

Chart 5 demonstrates the development of complement fixing antibodies in 3 calves. These calves were inoculated subcutaneously with 1.0 cc. of a 10^{-1} suspension of virulent spleen tissue. Calf 971 received the caprine strain; calf 973 the regular bovine strain; and calf 1001 the lapinized strain.[14] The calves were bled before the inoculation and at various intervals during the illness and subsequent recovery. Complement fixing antibodies appeared on the ninth day for 971, fourteenth day for 1001, and seventeenth day for 973. These results may account for the negative and suspicious reactions obtained with postvaccination serums from calves bled on the tenth, thirteenth, and fourteenth days.

PERSISTENCE OF COMPLEMENT FIXING ANTIBODIES

Calf 515 was vaccinated with the attenu-

CHART 4

Calves Vaccinated with Attenuated Avianized Vaccine										Calves Vaccinated with Inactivated Bovine Tissue Vaccine								
Calf No.	No. of days bled after vaccination	Result of challenge	Neutralization test	Amniotic and allantoic fluid antigen serum dilution 1:50						Calf No.	No. of days bled after vaccination	Result of challenge	Neutralization test	Amniotic and allantoic fluid antigen serum undiluted				
				Antigen Control	Serum control 1. cc.	1.0	0.4	0.2	0.1					Antigen control	Serum control 1. cc.	0.15	0.1	0.05
481	10th	I	+	—	—	—	—	—	—	295	14th	I	+	—	—	4+	3+	+
482	14th	I	+	—	—	4+	3+	2+	+	328	14th	I	+	—	—	4+	4+	+
498	14th	I	+	—	—	4+	4+	4+	4+	330	14th	I	+	—	—	4+	4+	4+
501	14th	I	+	—	—	4+	4+	4+	3+	402	14th	I	+	—	—	4+	4+	4+
514	14th	I	+	—	—	4+	3+	+	—	405	14th	I	+	—	—	4+	4+	3+
517	14th	I	+	—	—	4+	2+	—	—	463	14th	I	+	—	—	2+	+	—
519	14th	I	+	—	—	4+	4+	4+	4+	505*	14th	NI	?	—	—	4+	4+	4+
557	13th	I	+	—	—	+	—	—	—	667	14th	I	+	—	—	2+	+	—
565	13th	I	+	—	—	—	—	—	—	670	14th	I	+	—	—	4+	2+	—
572	14th	I	+	—	—	4+	4+	4+	4+	673	14th	I	+	—	—	4+	3+	2+
616	14th	I	+	—	—	4+	2+	+	—	721	14th	I	+	—	—	+	—	—
744	14th	NI	—	—	—	—	—	—	—	725	14th	I	+	—	—	4+	3+	+
792	14th	NI	—	—	—	+	±	—	—	743	14th	I	+	—	—	4+	4+	4+
795	14th	I	+	—	—	2+	+	±	—	752	14th	I	+	—	—	4+	4+	4+
797	14th	I	+	—	—	4+	3+	2+	+	811	14th	I	+	—	—	4+	4+	4+
802	14th	I	+	—	+	3+	2+	+	—	812	14th	I	+	—	—	2+	+	—
852	14th	I	+	—	—	4+	4+	3+	2+	813	14th	I	+	—	—	4+	4+	4+

I = Immune. NI = Non-immune * = Mildly ill.

ated avianized vaccine on 14 September 1944. The serum gave a positive reaction on the fourteenth day and remained positive, with a gradual decrease in titer, until approximately the 180th day. Eleven months after vaccination, the animal was bled and challenged with 1,000 to 10,000 m.i.d.'s of live virus. At this time, neutralizing but no complement-fixing antibodies were demonstrated. The animal proved to be immune on challenge. Serum collected twenty-two days following challenge was negative to the c.f. test.

Nakamura and Goto,[7] in reporting on the retention of complement-fixing antibodies in rabbits hyperimmunized with rinderpest, claimed the antibodies were present in two serums out of nine after the fourth month.

PRESERVATION OF COMPLEMENT AND SHEEP CELLS

The complement used for these investigations was obtained from 25 guinea pigs bled at six- to eight-week intervals. The serum was collected, pooled, and bottled in 2.0-cc. amounts in 60-cc. vaccine bottles and immediately placed in the freezer at —20 C. To date, the complement has been kept frozen from four to five months in this manner, without any loss of the original titer. The complement was bottled in small amounts because it was found that freezing and thawing two or three times reduced the hemolytic titer.

Desiccation of the complement from the frozen state was accomplished by two methods, one using the drum type of desiccator with dry ice refrigeration and the other with a mechanically refrigerated commercial apparatus. Twenty-five guinea pigs were bled, the serum pooled and bottled in 7.0-cc. amounts in 60-cc. vaccine bottles. Following dehydration the bottles were evacuated and stoppers inserted under vacuum. The product from the drum type of desiccator was similar to that purchased on the open market. To obtain a complement equal to the original titer, it was necessary to reduce the amount of diluent from 7.0 to 5.0 cc. The product obtained in the commercial desiccator reconstituted to

CHART 5

Calf No.	Date of bleeding	Date of challenge	No. days after challenge	Temp. of animal	Complement Fixation Test: Antigen control	Serum control .15	.15	.10	.05
971 Challenged with caprine strain of rinderpest virus.	15-IX-45	15-IX-45	0	103.2	—	—	—	—	—
	20-IX-45		5	105.6	—	—	—	—	—
	24-IX-45		9	102.0	—	—	+	±	—
	29-IX-45		14	101.8	—	—	2±	2+	4+*
	2- X-45		17	102.6	—	—	4+	4+	4+
973 Challenged with the original strain of rinderpest virus.	15-IX-45	15-IX-45	0	102.8	—	—	—	—	—
	20-IX-45		5	104.4	—	—	—	—	—
	24-IX-45		9	104.8	—	—	—	—	—
	29-IX-45		14	103.4	—	—	—	—	—
	2- X-45		17	102.6	—	—	2+	+	—
1001 Challenged with lapinized strain of rinderpest virus.	13-XI-45	13-XI-45	0	100.8	—	—	—	—	—
	21-XI-45		8	106.2	Sample hemolyzed. Discarded.				
	23-XI-45		10	104.0	—	—	—	—	—
	25-XI-45		12	101.4	—	—	—	—	—
	27-XI-45		14	101.2	—	—	2+	2±	—
	29-XI-45		16	101.6	—	—	4+	4+	4+
	9-XII-45		26	102.0	—	—	4+	4+	4+

*Zoning reaction—No. 971 serum enhanced hemolytic activity of complement.

the original volume after four to five months' storage appeared to have a titer equal to the original complement.

The sheep cells used in most of the tests were preserved in Alserver's solution, as suggested by Rein.[15] The cells were collected in an evacuated 300-cc. rubber-stoppered bottle containing 150 cc. of Alserver's

CHART 6—Effect of Time on Neutralization and Complement Fixation Tests Following Vaccination with an Avianized Vaccine

Calf 515—Vaccinated 14 Nov. 1944							
			Complement fixation test				
Date of bleeding	Neutralization test	Serum Control 0.15	Serum 0.15	Serum 0.10	Serum 0.05	Antigen control
14 Sept. 1944	—	—	—	—	—	—
28 Sept. 1944	+	—	4+	4+	4+	—
13 Oct. 1944	+	—	4+	4+	4+	—
14 Nov. 1944	+	—	4+	4+	4+	—
14 Jan. 1945	+	—	3+	2+	1+	—
9 Mar. 1945	+	—	+	±	—	—
14 May 1945	+	—	—	—	—	—
9 July 1945*	+	—	—	—	—	—
31 July 1945	+	—	—	—	—	—

*—Calf challenged with 10^{-1} dilution of spleen of calf 853.

blood preserving fluid. This solution contains dextrose 2.05 per cent, sodium chloride 0.42 per cent, and trisodium citrate 0.8 per cent, and is adjusted to a pH of 6.1 with citric acid. A 300-cc., rubber-stoppered bottle is filled to the 150-cc. mark, evacuated by suction, and sterilized for fifteen minutes at 15-lb. pressure. The animal is bled directly into this bottle and the contents thoroughly mixed. Then 30 to 35 cc. of the mixture is placed in 60-cc., sterile vaccine bottles for storage at 3 to 6 C. until ready for use. Whenever sheep cells are needed, a bottle of the suspension is removed from the refrigerator, strained through two thicknesses of cheesecloth, and washed in the usual manner. Cells are being used, which have been preserved for over sixty days with results which appear to equal those obtained with fresh cells.

DISCUSSION

The determination of the virus content in embryonating eggs by means of the c.f. test appears to be dependable after the virus has become sufficiently avianized. During the early transfers, an egg series is maintained by membrane passages. The concentration of virus in the fluids slowly increases to the point where fluids alone can be used for passage inoculum. Until the concentration of virus in the AA fluids becomes sufficiently high to permit fluid passage, *in vitro* tests may give results inconsistent with reactions *in vivo*. When the viral content of egg fluids, used as seed, exhibits a c.f. titer of 1:2 to 1:5, it was shown that AA fluids pooled from a large group of eggs had a titer usually equal to or better than the original seed inoculum. The viral titers of small groups of eggs appear to vary considerably. The production of avianized seed virus and methods of handling has been described.[13]

It appears likely that an antigen with a higher titer could be developed by harvesting the AA fluids and amniotic membranes, using the former as a diluent when preparing the amniotic membrane suspension. The amniotic membrane has been shown to contain a higher concentration of antigen than the allantoic and yolk sac membranes.

It should be noted that in several titrations made on the chorioallantoic and yolk sac membranes, inconsistent results were obtained. In some membranes, high concentrations of antigen were found while others were quite low. The irregularity of these results may have been due to the presence or absence of portions of the amniotic membrane. The amnion is closely associated with the other membranes and unless care is taken they are difficult to separate. A total of 302 antigen titrations were conducted on various amniotic and allantoic fluids and membranes as well as yolk sac fluids and membranes. Of this number, 200 induced positive reactions, 69 were negative, and 33 gave doubtful results. The negative and doubtful reactions resulted from testing AA fluids from early series of egg passages maintained by membrane transfers.

The use of the c.f. test for titrations and diagnostic purposes gave excellent results.

In comparing this test with the neutralization tests, the results are similar to those reported by Howitt,[5] who states that the former test is more reliable for serum obtained several months following the onset of a neurotropic virus disease. Howitt[5] also reported that serums stored over six months had a tendency to become anticomplementary. At this station, the serums from animals vaccinated against rinderpest were tested after storage from six to twenty months with satisfactory results. Zoning occurred in seven serums and appeared to be due to a complement stimulating factor in the serum.

While investigating the time required for the fixation of the complement by the antigen and the antibody, it was decided that eighteen to twenty-two hours at 4 to 7 C. was most satisfactory. Nakamura and Goto[7], with a lymph gland antigen, used a four-hour incubation at 4 to 7 C., but as can be observed in chart 3, a four-hour incubation period was not sufficient time for fixation with AA fluids as an antigen.

The enhancing property of AA fluids on the hemolytic activity of the complement appears to remain the same, whether the dose of antigen is 0.5 or 1.5 cc. The enhancing factor of the fluids may be due to ascorbic acid. King[16] reports that ascorbic acid is synthesized early in the developing embryo. The stimulating effect of ascorbic acid on complement activity and stability is reported by Ecker *et al.*[17] Craigie[18] has suggested that one or more of the components of the complement not affected by heat may be the source of the enhancing property. One of these factors may be responsible for the enhancement of complement by bovine serums. Desiccation of the frozen AA fluids reduces the enhancing property on complement.

The use of frozen complement and preserved sheep cells proved to be advantageous, especially when conducting the c.f. tests daily. Complement which has been kept frozen from four to five months gave the same titer, whether titrated with fresh or preserved cells.

SUMMARY

A satisfactory complement fixation test for rinderpest is reported. The antigen is easily produced in large quantities and readily standardized. The titer of the antigen is maintained for long periods when stored frozen. Heating the antigen for sixty minutes at 60 C. inactivates the virus, but the antigenicity is unimpaired. The test is of great value in measuring the virus content of eggs used for vaccine production, or seed virus for egg inoculation. Complement-fixing antibodies can be demonstrated regularly in the serum of vaccinated animals beginning approximately fourteen days after vaccination and persisting for five to six months.

The preservation of complement by freezing and the use of sheep cells preserved with Alserver's solution were found satisfactory.

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